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(54) Title: PROMOTION OF CNS REGENERATION BY OLFACTORY BULB GLIA

(57) Abstract

The present invention relates to "activated" astrocytes and the methods of utilizing the activated astrocytes as a means for promoting central nervous system nerve growth and regeneration, blood vessel growth and regeneration, and/or reducing secondary necrosis and glial scar formation. The activated astrocytes and pharmaceutical compositions comprising same, may be used to treat disorders of the nervous system resulting from trauma or diseases which have in some way damaged the nerve tissue.

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Promotion of CNS Regeneration by Olfactory Bulb Glia

1. INTRODUCTION

The present invention relates to "activated" immature astrocytes and the methods of utilizing the activated immature astrocytes as a means for reducing secondary necrosis and glial scar formation, promoting blood vessel and central nervous system axon growth and/or regeneration, and reforming or reinforcing the blood brain barrier. The activated immature astrocytes and pharmaceutical compositions comprising same, may be used to treat disorders of the nervous system resulting from trauma or diseases which have in some way damaged the nerve tissue.

2. BACKGROUND OF THE INVENTION

In the central nervous system, the chief non-neural cells are the glial cell types. These vary in numbers and type from one part of the nervous system to another, but the two basic classes can be distinguished by their size and embryonic origin, namely the macroglia, which are relatively large cells derived from the neural plate, and the smaller microglia which stem from the mesodermal tissues surrounding the nervous system.

The macroglia comprise two cell types, the astrocytes (astroglial cells) and the oligodendrocytes (oligodendroglial cells).

Astrocytes possess small cell bodies (the nucleus is about 8 microns in diameter in man) with ramifying dendrite-like extensions. The cytoplasmic

processes of astrocytes carry fine, foliate extensions which partly engulf and separate neurons and their neurites, and often end in plate-like expansions on blood vessels, ependyma and on the pial surface of the central nervous system.

The functions of astrocytes are numerous. They act mechanically as a supporting component of the nervous system. Their microfilaments, microtubules, and surface contact zones fit them for this task. They also act defensively by phagocytosing foreign material or cell debris. They can function as antigen presenting cells to macrophages and can provide a means of limited repair by forming glial scar tissue or filling the gaps left by degenerated neurons. In addition, they have essential metabolic functions in regulating the biochemical environment of neurons, providing nutrients, and regulating acid-base levels, etc.

Moreover, the astrocytes, which are able to divide in immature and mature animals, pass after mitosis through a series of structural transformations depending on their state of maturity. In areas of brain injury in young or old animals they proliferate (gliosis) to produce neural support. In a penetrating injury to the central nervous system (CNS) of adult mammals, severe tissue damage and secondary necrosis occurs in the region surrounding the wound. The degenerating effects caused by the injury are believed to generate a response in the surviving glial cells adjacent to the site of the injury (Reier et al., 1983, The Astrocytic Scar As an Impediment to Regeneration in the Central Nervous System, Spinal Cord Reconstruction, Raven Press, N.Y., pp. 163-195). The astrocyte response consists of a slight mitotic increase, an increase in size (hypertrophy), and a concomitant increase in quantity of intermediate filaments (Mathewson, et al., 1985, Brain Res. 327:61-69). Together with

invading monocytes, the astrocytes act as phagocytes to clear debris within the wound cavity (Schelper, et al., 1986, J. Neuropath. and Exper. Neurol. 45:1-19). When the injury disrupts the pial lining of the brain, fibroblasts migrate into the wound cavity and multiple layers of basal lamina form over the astrocyte surface (Bernstein, et al., 1985, Brain Res. 327:135-141). The fibroblasts also produce collagen, which forms dense bundles within the surrounding extracellular spaces several weeks after injury. Thus, in adults the astrocytes, together with other cellular elements, form dense interwoven scars which fill the space vacated by the dead or dying cells in the injury area. Although the scar may help save the organism it also blocks axonal regeneration and the individual is left with an irreversible functional deficit or epileptic focus depending on the site of the lesion.

Previous studies by the inventors and others indicated that penetrating lesions in the central nervous system (CNS) of neonatal mammals rarely resulted in the formation of glial scars similar to those observed in adults and that the production of typical adult glial scars after injury increased after the first two postnatal weeks in rodents (Barrett, et al., 1948, Exp. Neurol. 84:374-385; Smith, et al., 1986, J. Comp. Neurol. 251:23-43).

Previous studies on regeneration have demonstrated that CNS axons have the potential to grow long distances through peripheral nerve grafts (Friedman, et al., 1985, J. Neurosci. 5:1616-1625) or Schwann cell bridges (Kromer, et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:6330-6334). However, the studies with peripheral nerve elements indicated that regenerating nerve fibers could only extend a short distance upon reentry into the CNS, most likely due to the formation of scars at the ends of the graft. In addition, after

repeatedly crushing or cutting the dorsal roots near their entrance point in the spinal cord, the peripheral sensory fibers are regenerated only as far as the dorsal root entry zone (DREZ) of the spinal cord but no further. The problem at the DREZ is analogous to the failure of axon regeneration throughout the remainder of the CNS. Although the distance needed to reconnect the regenerating sensory fibers with their denervated dendrites in the dorsal horn of the spinal cord is relatively short (i.e., only fractions of a millimeter in the adult rat), this scant distance is normally never breached by regenerating sensory fibers in adult animals. Thus, although the injured adult CNS is potentially capable of a considerable amount of regeneration, sprouting is usually abortive and the axons fail to reinnervate their appropriate targets.

In addition, studies by the inventors indicated that developing axons are guided by oriented "highways of astroglial tissues" (Silver, et al., 1979, Dev. Biol. 68:175-190; Silver, et al., 1982, J. Comp. Neurol., 210:10-29).

The inventors have shown that in early postnatal lesion-induced acallosal animals, an untreated, properly shaped nitrocellulose (Millipore) filter, placed adjacent to the neuromas and spanning the lesioned cerebral midline, can support the migration of immature glia (Silver, et al., 1983, Science, 220:1067-1069).

In addition to scar formation, another undesirable consequence of injury to the nervous system is the development of edema. Cerebral trauma (including surgical trauma), infarction, abscess and hypoxia all can result in brain edema.

Klatzo classified different types of brain edema, on the basis of pathogenesis, into two categories, vasogenic edema and cytotoxic edema (Adams and Victor, 1985, in "Principles of Neurology"; Third Edition, McGraw-Hill Book Co., New York pp. 476-480).

Vasogenic edema is associated with increased permeability of capillary endothelial cells, and is associated with tumor growths and other localized processes as well as toxic injuries to blood vessels. Cytotoxic edema, in contrast, is associated with the swelling of cellular elements (neurons, glia, and endothelial cells); it is exemplified by the cellular response to hypoxic injury.

Because the brain is enclosed in a limited amount of space, edema (swelling) of the brain can result in pressure and displacement of brain tissue with consequent depression of cerebral function. Thus, a patient may become apathetical, drowsy, or confused and, if areas of the brain associated with vital functions are involved, the condition may be life threatening. Current methods of treating edema include the administration of osmotic agents such as mannitol which result in the removal of fluid from brain tissues and treatment with corticosteroids.

U.S. Patent No. 4,900,553 entitled "Method of Reducing Glial Scar Formation And Promoting Axon And Blood Vessel Growth And/Or Regeneration Through The Use Of Activated Immature Astrocytes" was filed on September 11, 1987 and issued on February 13, 1990.

3. SUMMARY OF THE INVENTION

The present invention is directed to "activated" astrocytes, pharmaceutical compositions comprising the same, and methods of utilizing the activated astrocytes to treat patients with damage to the nervous system.

According to the present invention, it has now been determined that c ntrary to normal adult astrocytes which promote glial scar formation, certain "critical period" activated astrocytes reduce glial scar formation, inhibit extensive bleeding and secondary necrosis, restore the integrity of the blood brain barrier, and promote central nervous system axonal regeneration. Thus, the activated astrocytes can be administered therapeutically, to promote axon regeneration in the central nervous system, to promote blood vessel regeneration, to reform or reinforce the blood brain barrier, and/or to reduce glial scar formation and necrosis.

One embodiment of the present invention concerns the use of "activated" astrocytes in injectable form or on implants to promote directed axon regeneration and reduce glial scar formation in the forebrain, or in damaged spinal axons of the central nervous system.

The invention is illustrated by way of examples infra, wherein activated immature astrocytes (murine postnatal day 8 or less) were harvested and transplanted into postcritical period animals (postnatal day 14 or more) in order to determine whether an environment conducive for axon regeneration could be re-established in the postcritical animals. The results (discussed in more detail below) indicate that the immature astrocytes survive implantation and reduce the deleterious sequelae of lesions in the brain. In addition, axonal regeneration and/or blood vessel growth was enhanced and the blood brain barrier was restored, as measured by impermeability to Evans-Blue dye. The beneficial effects observed included the reduction of necrosis and glial scar formation at the lesion site, as well as the stimulation and promotion of a substratum for the regeneration of postcritical period callosal axons that were not otherwise observed to regenerate. Although the initial studies were

performed with activated immature astrocytes (postnatal day 8 or less) harvested on millipore filters placed in the forebrain of postcritical period mice, further examples, presented herein, indicated that the same beneficial effects are observed when the activated astrocytes are implanted on polymers in the spinal cord region of paralyzed animals.

In a further embodiment of the invention, cells having the properties of activated immature astrocytes (ensheathing cells) may be obtained from the olfactory bulb of immature or mature humans or animals. Such olfactory bulb cells have been found to be capable of producing an especially favorable axon growth-promoting surface.

In addition, the activated astrocytes have been isolated, harvested, and purified in vitro for use in vivo as well as in vitro. In addition, because normal immature astrocytes mature in culture, purified activated immature astrocytes have been genetically engineered to be immortal and forever immature for in vivo therapeutic use. Similarly, the activated astrocytes have been used in vitro to promote axon growth in biological cultures.

3.1. DEFINITIONS

As used herein, the following terms shall have the meanings indicated:

BSA	=	bovine serum albumin
CMF	=	calcium-, magnesium-free buffer
CNS	=	central nervous system
DNase	=	deoxyribonuclease
DMEM	=	Dulbecco's modified Eagle's medium
DREZ	=	dorsal root entry zone
FCS	=	fetal calf serum
GFAP	=	glial fibrillary acid protein

HRP	=	horseradish peroxidase
Ig	=	immunoglobulin
NGS	=	normal goat serum

4. DESCRIPTION OF THE FIGURES

FIGURES 1a, 1b, 1c, and 1d are scanning electron micrographs of acallosal mice implanted on postnatal day 2 and examined 24 hours later. In FIGURE 1a, a brain viewed from above shows a filter (I) that was placed horizontally across the midline extending into each cortical hemisphere. Higher magnification of the surface of the filter showing many attached cells is illustrated in FIGURE 1b. As shown in FIGURE 1c, the first axons (arrowheads) to extend across the filter do so nonfasciculated and along the glia that have attached to the implant. FIGURE 1d shows that astrocytes respond to the presence of axons (Ax) by extending small processes (arrow) that encircle the axons. The magnification of the respective FIGURES is as follows: (a) x20; (b) x700; (c) x2,000; (d) x16,000.

FIGURES 2a, 2b, 2c, 2d, and 2e are micrographs of coronal sections through the nitrocellulose bridge (I) and associated tissue of the hemispheric midline of acallosal mice. FIGURES 2a-2c represent animals implanted at "critical" stages; postnatal days 2 (FIGURE 2a) and 8 (FIGURES 2b, 2c), both examined 48 hours after implantation. A comparison of FIGURES 2a-2c to animals implanted at "postcritical" stages, 14 (FIGURE 2d) and 21 (FIGURE 2e) days after birth, both examined 7 days postoperatively, indicate that in FIGURES 2a-2c the glia are more stellate, sending many cytoplasmic processes into the pores of the filter, whereas the cells near the implant in FIGURE 2d and FIGURE 2e appear flat, lacking extensive infiltration of processes. Directly above the infiltrated stellate glia of "critical" period implants

are numerous axons (asterisks; FIGURES 2a-2c), but such axons were not apparent in "postcritical" stage implants (FIGURES 2d-2e). The magnification of the respective FIGURES is as follows: (a) x400; (b) x125; (c) x400; (d) x400; (e) x400.

FIGURES 3a, 3b, 3c, 3d, and 3e are micrographs of the corpus callosum of a previously acallosal animal implanted with a Millipore implant on postnatal day 5. When the animal was killed five weeks later, a new callosum (CC) had formed above the implant (FIGURES 3b and 3c). Further rostrally the bridge did not span the midline and was embedded in only one of the neuromas (FIGURE 3a). In this region small groups of axons left the neuroma but grew ectopically along the subependymal zone within the dorsal septum (*). Uniquely, the animal had both large longitudinal neuromas (LB) and a well-developed callosum in the same plane of section as well as the ectopic ipsilateral septal projection (FIGURES 3b and 3c). This uniqueness provides a marker that ensures that the animal was acallosal at the time of implantation. Horseradish peroxidase injected into the cortex of one hemisphere in the region of the de-novo-formed callosum (arrow) labels neurons on the opposite side of the brain (bracketed area of FIGURE 3c; higher magnification, FIGURES 3d and 3e). The reformed callosum has grown to its appropriate region of synaptic termination (small arrows). The magnification of the respective FIGURES is as follows: (a) x100; (b) x100; (c) x100; (d) x250; (e) x400.

FIGURES 4a, 4b, and 4c are micrographs of coronal sections through filters implanted into postnatal day 2 acallosal mice and examined 24 (FIGURE 4a), 48 (FIGURE 4b), and 72 (FIGURE 4c) hours later. In FIGURE 4a, twenty-four hours after implantation, numerous glia (arrowheads), some of which are phagocytic (insert),

have migrated out of the hemisphere and along the implant. As they attach to the implant (I) they extend cytoplasmic processes into the pores of the filter (small arrows in FIGURES 4b and 4c); note that the leading glial cell (far right) has extended few processes. In FIGURE 4b, within 48 hours, glia coat the majority of the filter surface, providing a substrate on which axons (Ax) and blood vessels have extended. As shown in FIGURE 4c, in some specimens 72 hours after implantation, the axons fasciculate over the glia above the filter, a configuration similar to that of the normal developing corpus callosum and "sling". Note in FIGURE 4c however, the absence of a glial limiting membrane. The magnification of the respective FIGURES is as follows: (a) x500; (b) x400; (c) x500; insert x4,400.

FIGURES 5a and 5b are micrographs of coronal sections illustrating the staining pattern for antibodies against GFAP in animals implanted at critical and postcritical ages. In FIGURE 5a, acallosal neonates (P2) implanted with filters and examined after five days show extensive astrocytic processes within the implant (I) and within the cortex (Cx), retaining their stellate morphology. In FIGURE 5b, the astrocytes in acallosal animals implanted at postcritical stages (P21) and examined after one week appear flat within the scar above the implant (I). The magnification of the respective FIGURES is as follows: (a) x300; (b) x300.

FIGURES 6a, 6b, 6c, 6d, 6e, and 6f are micrographs of coronal sections showing the staining pattern produced by antibodies against laminin protein. As shown in FIGURE 6a, in critically implanted before P8 animals laminin not only appears to be confined to the basal lamina of blood vessels and the pia, but it is also along glial processes (arrows) within the filter (FIGURES 6a and 6b). When animals were implanted at postcritical

stage (P21) antilaminin stained the basal lamina in the scar that extends around the implant (I) and appears continuous with the longitudinal fissure (LF; FIGURES 6c and 6d). The cells producing the laminin are flat. Post-critical period animals P34 (FIGURES 6e, 6f) implanted with glial-coated filters from P2 neonates show no scar formation and thus, a laminin staining pattern identical to critical period animals given naked implants alone (compare FIGURES 6a and 6b to FIGURES 6e and 6f). The magnification of the respective FIGURES is as follows (a) x100; (b) x250; (c) x100; (d) x250; (e) x100; (f) x250.

FIGURES 7a, 7b, 7c, and 7d are transmission electron micrographs of acallosal mice implanted 8 days after birth and killed 48 hours later. In FIGURE 7a, glia attached to the implant have a stellate morphology; microglia (M) are also apparent. Among and above the glia that have sent processes into the filter (arrows) are axons (Ax) and blood vessels (BV). As noted in FIGURE 7b, the axons (small arrows) that extend into areas where basal lamina (BL arrowheads) appear are positioned immediately adjacent to the glia but not to the basal lamina. Higher magnification in FIGURES 7c and 7d shows axons associated with astrocyte processes (G) containing intermediate filaments and glycogen granules. The magnification of the respective FIGURES is as follows: (a) x4,500; (b) x5,000; (c) x12,000, and (d) x12,000.

FIGURES 8a, 8b, 8c, 8d, 8e, and 8f are scanning electron micrographs of acallosal mice implanted on postnatal day 2 with partially crushed filters and examined 48 hours later. In FIGURE 8a, the glia above the crushed portion of the filter (C) are flat and have a smooth surface (FIGURES 8a and 8b), whereas the glia attached to the noncrushed area (NC) are more stellate in shape (FIGURES 8a, 8b, and 8f). In places, some of the

flat glia over the crushed implant, rippled and extended many very short processes (FIGURE 8d). In areas where this occurred, other glia moved onto the flattened cells establishing a cellular pile (FIGURE 8e). The magnification of the respective FIGURES is as follows: (a) x200; (b) x1,800; (c) x600; (d) x3,500; (e) x1900; (f) x2,100.

FIGURES 9a and 9b are micrographs of coronal sections of postnatal day 27 (P27) animals that received transplanted filters precoated with glia from neonates that were injected with ^{3}H-thymidine. In FIGURE 9a, a majority of the glia attached to the filters labeled with silver grains over the nuclei. Cells further removed from the implant surface and along blood vessels are also labeled by ^{3}H-thymidine (arrows). Transplanted neonatal glia on Millipore (I) placed into post-critical-period animals (P34) retain their stellate morphology, as shown when stained with antibodies against GFAP (see FIGURE 9b). The magnification of the respective FIGURES is as follows: (a) x450; (b) x300.

FIGURES 10a, 10b, 10c, and 10d are micrographs of coronal sections of untreated implants placed into postcritical acallosal animals (FIGURES 10a and 10b) and transplanted filters precoated with glia harvested from neonates (FIGURES 10c and 10d). The reacting cells along the untreated filter (FIGURES 10a and 10b), are arranged in sheets and have a flattened morphology, with a few processes extending into the implant. In contrast, the gliotic reaction produced in the postcritical brain by the transplant resembles critical period implanted animals. Numerous inserted processes (arrowheads) from stellate cells and minimal scar formation or necrosis are evident (FIGURES 10c and 10d). The magnification of the respective FIGURES is as follows: (a) x125; (b) x400; (c) x125; (d) x400.

FIGURE 11 is a transmission electron micrograph of the host/donor interface (far lateral to the midline) from a P17 acallosal animal that was given a precoated glial implant (transplant) and examined after six days. The astrocytes attached to the implant (I) retain their inserted processes which are rich in intermediate filaments (arrow). The cortex above the attached glia shows little tissue degeneration and no scar formation. The magnification in FIGURE 11 is x 12,600.

FIGURES 12a, 12b, 12c, and 12d are transmission electron micrographs at the midline of a postnatal day 17 acallosal mouse that received a pre-glial coated implant (I) from a 2-day neonate donor and was then examined six days after it resided in the host. As shown in FIGURE 12a, the glia attached to the implant have retained their stellate morphology and infiltrated cytoplasmic processes. Scarring and ectopic basal lamina are absent. Among the glia are many de-novo-formed axon bundles (arrowheads). Higher magnification shows loosely fasciculated unmyelinated axons (FIGURE 12b) and others (arrow) adjacent to astrocytic processes. Two daughters cells (D) above the implant share a midbody (open arrow in FIGURE 12d). Thus, transplanted cells can divide. The magnification of the respective FIGURES is as follows: (a) x10,000; (b) x20,000; (c) x10,000; (d) x 3,300.

FIGURES 13a, 13b, 13c, 13d, 13e, and 13f are micrographs of GFAP immunofluorescence. These micrographs show that both immature (FIGURE 13a) and mature (FIGURE 13b) astrocyte coated implants (I) have GFAP positive astrocytes attached to their surface. Astrocytes were purified and allowed to age in vitro. Photomicrographs of laminin immunoreactivity in P60 acallosal mice transplanted with either immature (FIGURE 13c) or mature (FIGURE 13d) astrocyte coated implants. Note the lack of basal lamina staining (i.e. scarring) in the host brain

receiving immature astrocytes (bracket in FIGURE 13c). However, basal lamina is quite apparent in host brains receiving mature astrocyte coated implants (bracket in FIGURE 13d). Basal lamina staining on the bottom of all transplants is caused by the lack of transplanted astrocytes in that portion since they were seeded on the top of the filters in culture. Autoradiograph of astrocytes transplanted into P60 mice which were labeled with ^{3}H-thymidine in culture. Labeled immature astrocytes (arrows) have the ability to migrate away from the surface of the implant (FIGURE 13e). Labeled mature astrocytes (arrowheads) did not appear to migrate away from the surface in the implant (FIGURE 13f). The magnification of the respective FIGURES is as follows: (a) x400; (b) x400; (c) x300; (d) x300; (e) x400; (f) x400.

FIGURES 14a and 14b are photomicrographs of immature (FIGURE 14a) and mature (FIGURE 14b) astrocyte coated filters (I) which were implanted into P60 acallosal mice. Host brains of animals implanted with immature astrocytes (culture 4 days) had minimal scar formation and tissue degeneration (area in bracket in FIGURE 14a). In contrast, the site of injury around implants coated with mature astrocytes (28 or more days in culture) developed a glial-mesenchymal scar similar to that observed in adult mice implanted with untreated filters (area in bracket in FIGURE 14b). The magnification of the respective FIGURES is as follows: (a) x400; (b) x400.

FIGURES 15a and 15b are transmission electron micrographs of the surface of an immature astrocyte coated implant (I) which was transplanted into a P60 acallosal mouse. Astrocytes containing intermediate filaments (gf) cover much of the surface of the implant (FIGURES 15a and 15b). However, there still exists a few monocytes (M) and small pockets of basal laminae (arrowheads). In the region of the cortex above the implant are intacted

neutrophils containing unmyelinated and myelinated axons (arrows). Transmission electron micrograph of the scar that formed in response to the transplantation of a mature astrocyte coated implant (I) into a P60 acallosal mouse. The glial-mesenchymal scar contains collagen (cf), fibroblasts (large arrowheads) and basal laminae (small arrowheads) and fibroblasts (FIGURE 15b). The magnification of the respective FIGURES is as follows: (a) x4,400; (b) x4,400.

FIGURES 16a and 16b are scanning electron micrographs of axons extending over the glia (asterisk) attached to a filter implanted into an acallosal postnatal (day 2) and examined 48 hours later. The low power insert (FIGURE 16a) shows callosal axons extending from only one hemisphere, out of the neuroma (LB) and across the implant (I). The caudal tip (CT) and borders of the filter are apparent. Higher magnification (FIGURE 16b) shows large fascicles of axons traversing the implant towards the opposite hemisphere. However, not all axons retain their orientation and some wander in the middle of the filter (arrows). The magnification of the respective FIGURES is as follows: (a) x80; (b) x700.

FIGURES 17a, 17b, and 17c are micrographs of coronal sections through a partially crushed filter implanted into a postnatal day 2 acallosal mouse and sacrificed 48 hours later. In FIGURE 17a, the axons (Ax) traverse the surface of the implant along the stellate "activated" glia (i.e. inserted) that extend processes into the non-crushed portion of the filter (NC), but they turn abruptly at the crush (C)/non-crush (NC) interface. The astrocytes attached to the surface of the crushed portion were non-activated flattened (arrowheads and upper insert), displayed a mound where the nuclei reside and had lamellipodia (L in second insert) at their periphery. As noted in FIGURE 17b, the axon bundle (Ax) turns

perpendicularly and travels along the crush/non-crush interface. In FIGURE 17c, fibers crossed over the crushed portion of the implant above a mat of glia with a flat-celled bottom layer (open arrow). The fibers then grew rostrally on another inserted group of cells on the other side of the crush (Ax at the right in FIGURE 17b). Some axons were also present within the filter on the inserted activated astroglial processes. The magnification of the respective FIGURES is as follows: (a) x400; (inserts) x625; (b) x3,300; (c) x400.

FIGURES 18a, 18b, and 18c are schematic drawings demonstrating the placement of the "pennant-shaped" nitrocellulose implant in the dorsal root entry zone of the spinal cord. FIGURE 18a is a frontal view of the "pennant-shaped" nitrocellulose implant (10) containing the activated immature astrocytes (12). FIGURE 18b is a cross sectional view of the "pennant-shaped" implant (10) coated with the activated immature astrocytes (12) inserted into the dorsal root cord interface. The pole portion (14) of the pennant protrudes outside the spinal cord (18) into the dorsal root (20) itself, while the broad portion (16) of the pennant-shaped implant lays in the cord (18) proper. FIGURE 18c is an overhead view of the "pennant-shaped" implant (10) coated with the activated immature astrocytes inserted into the dorsal root (20) cord (18) interface.

FIGURES 19a, 19b, 19c, and 19d are micrographs showing the placement of the "pennant-shaped" nitrocellulose implant in the L5 dorsal root entry zone of the spinal cord of a 180 day or older rat. FIGURES 19a through 19c indicate that the combination of embryonic astrocytes plus the oriented nitrocellulose implant, represses scar formation locally in the L5 dorsal root entry zone (FIGURE 19b) and stimulates axons and blood vessels to enter the central nervous system along the

implant surface (FIGURE 19b) and stimulate axons and blood vessels to enter the central nervous system along the implant surface (FIGURES 19a and 19c). FIGURE 19d demonstrates the normal scar formation which occurs at the dorsal root entry zone of a root-lesioned rat. As indicated in FIGURE 19d, when no nitrocellulose implant coated with activated immature astrocytes was inserted in the dorsal root-cord interface, no axonal regeneration was observed. The magnification of the respective FIGURES is as follows: (a) x400; (b) x25; (c) x60; (d) x400.

FIGURE 20. Micrographs of the three morphologically distinct types of E-18 forebrain neurons grown on the surface of immature and mature astrocyte monolayers for 16 hours. a) Neuron lacking any characteristic axon with only multiple short neurites. b) Neuron with one medium length axon-like neurite and multiple short neurites. c) Neuron with one long axon-like neurite and multiple short neurites. All cells were surface labelled with Texas red-conjugated-tetanus toxin C fragment and visualized under rhodamine optics. Scale bar = 10 μm.

FIGURE 21. Neurite outgrowth over the surface of either immature (a,c, and e) or mature (b,d, and f) rat forebrain astrocytes. E-18 neurons were grown for 16 hours and then labelled with tetanus toxin C fragment. After fixation in acid alcohol, cultures were labelled with anti-GFAP serum followed by G anti-Rig-FL. The cultures were viewed with (a and b) Rhodamine, (c and d) fluorescein, and (e and f) phase contrast optics. Note that neurite outgrowth is more extensive over the surface of the immature astrocytes than over the surface of the mature astrocytes (compare the labelled cells in a and b), and that the immature astrocytes have a more variable expression of GFAP than the mature astrocytes. Scale bar = 20 μm.

FIGURE 22. Neurite outgrowth on immature (closed diamonds) or mature (open squares) astrocytes over different periods of time in culture. Note that in both cases the rate of neurite outgrowth is relatively constant over the time course of the experiment. However, the overall rate of neurite outgrowth is more rapid on immature compared to mature astrocytes.

FIGURE 23. Adhesion of E-18 forebrain neurons to the surface of immature (a and c) and mature (b and d) astrocyte cultures. Neurons labelled in suspension with fluorescein diacetate were allowed to adhere to the astrocyte surface for 30 minutes, and non-adherent cells were removed. Cultures were viewed with fluorescein (a and b) or phase contrast optics (c and d). Note that many more neurons adhere to the surface of immature astrocytes than to the surface of mature astrocytes. Scale bar = 50 μm.

FIGURE 24. Transmission electron micrographs of cultured immature (a and b) and mature (c and d) astrocytes. Note the immature astrocytes (a) are smaller, contain a higher density of organelles but fewer intermediate filaments (b) when compared to mature astrocytes. Scale bar = 10 μm in a and c; 1 μm in b and d.

FIGURE 25. Frozen sections of adult rat CNS that had received either immature (a and b) or mature (c and d) astrocyte coated implants. Sections were double labelled by indirect immunofluorescence with antibodies against GFAP (a and c) and laminin (b and d). Virtually no gliotic response occurs around the immature astrocyte coated implant, which has become well integrated into the host CNS. By contrast, a substantial glial scar is seen in the region of the mature astrocyte coated implant. (i = implant). Bar = 250μm.

FIGURE 26. Frozen sections of adult rat CNS that had received implants coated with immature astrocytes containing FITC labeled microspheres. In (a) and (b), a section labelled with antibodies to GFAP followed by G-anti-RIg-RITC and viewed in the rhodamine channel in (a) and fluorescein channel in (b). The FITC containing cells label with anti-GFAP and are found on the surface of the implant and in the surrounding tissue. In (c) and (d), a section labelled with antibodies to laminin as above. Note that the majority of FITC labelled astrocytes are associated with laminin-reactive blood vessels in the CNS. Lower panel, higher magnification of GFAP labelled section examined in phase, (e) rhodamine, (f) and fluorescein (g) optics. The FITC containing astrocytes on the implant surface have processes which penetrate the implant while those in the surrounding tissue are associated with blood vessels. (i = implant) Bar = 100μm in a-d and 60μm in e-g.

FIGURE 27. Electron micrographs from adult rat CNS that received implants coated with colloidal gold labelled immature astrocytes. Low magnification (a) and (b) of lightly labelled astrocytes associated with blood vessels in the host CNS. Higher magnification (c) showing the vesicular location of the gold label. In (d) a labelled astrocyte process in the region of the implant appears associated with two different blood vessels and (e) higher magnification of the same region showing close association between labelled astrocyte process and blood vessel endothelial cell. (a = astrocytes, b = blood vessels, e = endothelial cell). Arrows indicate colloidal gold containing organelles. Bars = 5μm in (a) and (d), 3μm in (b) and 1.5μm in (c) and (e).

FIGURE 28. Electron micrographs from adult rat CNS that had received implants coated with colloidal gold labelled mature astrocytes. Low magnification micrograph (a) showing the heavily labelled mature astrocytes

associated with the surface of the implant. Note that many of the astrocytes have a flattened morphology and that the implant is surrounded by a glial scar. Macrophages (b) and (c) in the region of the implant frequently contained colloidal gold label (arrow in b) and astrocyte derived cellular debris (arrow in c). (i = implant, a = astrocytes, SC = glial scar). Bars = 10μm in (a), 5μm in (b) and 1μm in (c).

FIGURE 29. Frozen sections from adult rat CNS that had received implants coated with either immature, (a) and (b) or mature (c) and (d) type-1 astrocytes, and 9 days later were sacrificed 4 hours after receiving a systemic injection of Evans Blue as described in the methods. Sections viewed under either phase (a, c) or fluorescein (b, d) optics. Note that although some preparation damage is present around the immature astrocyte coated implant the blood vessels in that region are impermeable to the dye. By contrast, in the region of the implant coated with mature astrocytes the blood vessels are permeable to the systemically applied tracer. (i = implant). Bar = 100μm

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to "activated" astrocytes, pharmaceutical compositions comprising the same, and methods of utilizing the activated astrocytes to treat patients with damage to the nervous system. According to the present invention, "activated" astrocytes is construed to refer to cells which are capable of producing a favorable axon growth-promoting surface, and includes but is not limited to immature astrocytes derived from organisms at a critical period of development or, alternatively, may be olfactory bulb astrocyte/glial cells or ensheathing cells.

According to the present invention, it has now been determined that contrary to normal adult astrocytes which promote glial scar formation, certain "critical period" or other activated astrocytes (e.g. olfactory glia) reduce glial scar formation, inhibit extensive bleeding and secondary necrosis, restore the integrity of the blood brain barrier and promote central nervous system axonal regeneration. Thus, the activated astrocytes can be administered therapeutically, to promote axon regeneration in the central nervous system, to promote blood vessel regeneration, to reform or reinforce the blood brain barrier and/or to reduce glial scar formation and necrosis.

One embodiment of the present invention concerns the use of "activated" astrocytes in injectable form or on implants to promote directed axon regeneration and reduce glial scar formation in the forebrain, or in damaged spinal axons of the central nervous system.

The invention is illustrated by way of examples infra, wherein activated immature astrocytes (murine postnatal day 8 or less) were harvested and transplanted into postcritical period animals (postnatal day 14 or more) in order to determine whether an environment conducive for axon regeneration could be re-established in the postcritical animals. The results (discussed in more detail below) indicate that the immature astrocytes survive implantation and reduce the deleterious sequelae of lesions in the brain. In addition, axonal regeneration and/or blood vessel growth was enhanced and the blood brain barrier was restored, as measured by impermeability to Evans-Blue dye. The beneficial effects observed included the reduction of necrosis and glial scar formation at the lesion site, as well as the stimulation and promotion of a substratum for the regeneration of postcritical period callosal axons that were not otherwise

observed to regenerate. Although the initial studies were performed with activated immature astrocytes (postnatal day 8 or less) harvested on millipore filters placed in the forebrain of postcritical period mice, further examples, presented herein, indicated that the same beneficial effects are observed when the activated immature astrocytes are implanted on polymers in the spinal cord region of paralyzed animals.

In addition, the activated immature astrocytes have been isolated, harvested, and purified in vitro for use in vivo as well as in vitro. In addition, because normal immature astrocytes mature in culture, purified activated immature astrocytes have been genetically engineered to be immortal and forever immature or activated for in vivo therapeutic use. Similarly, the activated immature astrocytes have been used in vitro to promote axon growth in biological cultures.

5.1. ACTIVATED ASTROCYTES

The present invention relates to activated astrocytes and the uses thereof to reduce scar formation and promote central nervous system nerve, and/or blood vessel growth or regeneration in areas of nerve damage. Nerve damage should be construed to refer to any damage to nerve cells or supporting cells of the nervous system, including but not limited to damage caused by injury, surgery, infection, hypoxia, malignancy, metabolic disorder, toxic disorder, or hereditary condition. The activated astrocytes of the invention have the properties of early-stage, immature, astrocytes that have the ability to promote such growth and regeneration and reduce scar formation.

In one embodiment of the invention, such activated astrocytes for use in the invention can be identified by observing their ability, when seeded onto a suitable polymer implant and introduced in vivo, to promote nerve regeneration (see Sections 6, 7, infra).

In another embodiment, activated astrocytes can be identified for use by observing their ability, in vitro, to support increased CNS neurite outgrowth or neuronal adhesion relative to mature (e.g., isolated from an adult, or aged in vitro) astrocytes. As an example, assays such as those described in Section 9, infra, can be used.

In yet another embodiment, activated astrocytes can be identified morphologically. Activated astrocytes include but are not limited to those with a polygonal, epithelioid, or stellate, rather than flat, morphology. In addition, an abundance of condensed heterochromatin in the nucleus, and a dense cytoplasm containing many closely packed organelles, few dense bodies, and small numbers of intermediate filaments are characteristic of some activated astrocytes. In addition, activated astrocytes are usually small, with few junctional specializations, and a small cytoplasm to nucleus ratio. In contrast, inactive (e.g. mature) astrocytes often have a less dense cytoplasm with less closely packed organelles, but many dense bodies and large numbers of intermediate filaments. Inactive (e.g. mature) astrocytes also tend to be large cells, with little condensed chromatin and an increase in both size and cytoplasm to nuclear ratio, in close apposition with one another, and containing many junctional specializations between them. The above description is not meant to limit the invention, but rather, to provide a method for use in a specific embodiment of identifying a subset of activated astrocytes by virtue of their morphological characteristics.

The activated astrocytes of the invention are those astrocytes having properties associated with a critical period of development in the host from which they are derived. In one embodiment, such a critical time period can be identified as the time prior to full development of the neural pathways in a given region in the embryo or neonate. For example, in a specific embodiment in which it is desired to promote nerve regeneration in a specific region of the central nervous system, activated astrocytes for use can be selected by obtaining those astrocytes corresponding to a time period prior to the full development of the neural pathways in such specific region. For example, it is expected that the critical period for use in promoting regeneration and reducing scar formation in the area of the corpus callosum in the forebrain will end at a later stage than such critical period for use in the spinal cord, since the corpus callosum finishes developing at a later time than the neural pathways of the spinal cord. The time periods for neural pathway development are known in the art (see, e.g., Silver et al., 1982, J. Comp. Neurol. 210:10 (for rodent); Warkany, J., 1971, Congenital Malformations, Yearbook Medical Publishers, Inc., Chicago, Illinois, pp. 189-295 (for human); Hamilton, W. J., et al., 1972, Hamilton, Boyd and Mossman's Human Embryology; Prenatal Development of Form and Function, Heffer, Cambridge (for human)). Astrocytes selected for use in the present invention, initially derived from host organisms before completion of neural pathway development, may be tested for their activity in promoting CNS nerve regeneration and reducing scar formation by in vitro and/or in vivo assays as described infra in Sections 6-9. The astrocytes can be purified by standard procedures known in the art (see, e.g., Sections 7.1.1 and 9.1.1, infra).

In an embodiment in which murine astrocytes are used, the activated astrocytes of the invention consist of those astrocytes of about postnatal day 8 or less.

In an embodiment directed to the use of human activated astrocytes, such activated astrocytes can be obtained from the human embryo, and in a preferred embodiment, from embryos of the first trimester of pregnancy. For example, such astrocytes may be obtained from organ tissue donors, cryopreserved samples or immortalized cell lines derived therefrom, etc.

In another embodiment directed to the use of human activated astrocytes, such astrocytes/glial cells can be obtained from the olfactory bulb of human embryos, children or adults. Such olfactory bulb glial cells have the unique property, corresponding to "critical period" astrocytes from other regions of the central nervous system, of being capable of producing an especially favorable axon growth-promoting surface. These olfactory bulb glial cells can be obtained during standard neurosurgical procedures; for example, cells of the olfactory bulb are often removed in part during certain operations such as the clipping of an anterior communicating artery aneurism (see, e.g., Neurosurgery, 1984, Wilkins and Rengachary, eds., McGraw Hill). In a preferred embodiment, olfactory bulb ensheathing cells can be used.

Activated astrocytes for use in the present invention may be obtained directly from the host organism within the critical time periods. Alternatively, other sources of such activated astrocytes include but are not limited to immortalized activated astrocyte cell lines and/or cryopreserved samples containing activated immature astrocytes.

For example, activated astrocytes may be immortalized by procedures known in the art, so as to preserve sources of such astrocytes for future use. Immortalized astrocytes can be maintained in vitro indefinitely. Various methods of immortalization are known in the art and can be used in the practice of the instant invention, including but not limited to viral transformation (e.g., with SV40, polyoma, RNA or DNA tumor viruses, Epstein Barr Virus, bovine papilloma virus, or a gene product thereof), chemical mutagenesis, etc. In preferred embodiments, the cell line is immortalized by a virus defective in replication, or is immortalized solely by expression of a transforming virus gene product. For example, activated astrocytes can be transformed by recombinant expression vectors which provide for the expression of a replication-defective transforming virus or gene product thereof. Such procedures are known in the art.

As mentioned supra, in an alternative embodiment, activated astrocytes can be cryopreserved for use at some future time. Various methods for cryopreservation of viable cells are known and can be used (see, e.g., Mazur, 1977, Cryobiology 14:251-272; Livesey and Linner, 1987, Nature 327:255; Linner, J.G., et al., 1986, J. Histochem. Cytochem. 34(9):1123-1135; U.S. Patent No. 4,199,022 by Senkan et al.; U.S. Patent No. 3,753,357 by Schwartz; U.S. Patent No. 4,559,298 by Fahy).

In a preferred aspect of cryopreservation, cryoprotective agents, a controlled freezing rate, and storage at a low temperature such as that of liquid nitrogen (-196°C) can be used. Cryoprotective agents which can be used include but are not limited to dimethyl sulfoxide (DMSO) (Lovelock, J.E. and Bishop, M.W.H., 1959, Nature 183:1394-1395; Ashwood-Smith, M.J., 1961, Nature 190:1204-1205), glycerol, polyvinylpyrrolidine (Rinfret,

A.P., 1960, Ann. N.Y. Acad. Sci. 85:576), polyethylene glycol (Sloviter, H.A. and Ravdin, R.G., 1962, Nature 196:548), albumin, dextran, sucrose, ethylene glycol, i-erythritol, D-ribitol, D-mannitol (Rowe, A.W., et al., 1962, Fed. Proc. 21:157), D-sorbitol, i-inositol, D-lactose, choline chloride (Bender, M.A., et al., 1960, J. Appl. Physiol. 15:520), amino acids (Phan The Tran and Bender, M.A., 1960, Exp. Cell Res. 20:651), methanol, acetamide, glycerol monoacetate (Lovelock, J.E., 1954, Biochem. J. 56:265), and inorganic salts (Phan The Tran and Bender, M.A., 1960, Proc. Soc. Exp. Biol. Med. 104:388; Phan The Tran and Bender, M.A., 1961, in Radiobiology, Proceedings of the Third Australian Conference on Radiobiology, Ilbery, P.L.T., ed., Butterworth, London, p. 59).

A controlled slow cooling rate is preferred. Different cryoprotective agents (Rapatz, G., et al., 1968, Cryobiology 5(12):18-25) and different cell types have different optimal cooling rates (see e.g., Rowe, A.W. and Rinfret, A.P. 1962, Blood 20:636; Rowe, A.W., 1966, Cryobiology 3(1):12-18; Lewis, J.P., et al., 1967, Transfusion 7(1):17-32; Mazur, P., 1970, Science 168:939-949 for effects of cooling velocity). A programmable freezing apparatus, as one example, can be used.

Cryopreserved astrocytes are preferably thawed quickly (e.g., in a 37°C waterbath) before use according to the present invention in the promotion of nerve and blood vessel regeneration and/or reduction of scar formation.

5.2. METHODS OF USING ACTIVATED IMMATURE ASTROCYTES IN THE PROMOTION OF NERVE OR BLOOD VESSEL REGENERATION AND/OR SCAR REDUCTION

5.2.1. TREATMENT OF NERVE INJURY AND DISORDERS

Activated astrocytes can be used in accordance with the present inv ntion to treat subjects in which it is desired to promote CNS nerve or blood vessel regeneration, to reform or reinforce the blood brain barrier and/or reduce scar formation. Thus, activated astrocytes can be applied, in any of various formulations as described infra, to areas of nerve damage. The activated astrocytes can be administered to patients in whom the nervous system has been damaged by trauma, surgery, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, toxic agents, paraneoplastic syndromes, degenerative disorders of the nervous system, etc. Examples of such disorders include, but are not limited to, Alzheimer's Disease, Parkinson's Disease, Huntington's chorea, amyotrophic lateral sclerosis, progressive supranuclear palsy, and peripheral neuropathies. In another embodiment, activated astrocytes can be applied to a wound to reduce scar formation. As one example, after an operation, activated astrocytes can be applied according to the present invention in order to reduce scar formation from lesions due e.g., to arterio-venous malformation, necrosis, bleeding, craniotomy, which can secondarily give rise to epilepsy. In another embodiment of the invention, activated astrocytes can be used for treatment of epilepsy, by stabilizing the epileptic focus and reducing scar formation.

In preferred embodiments of the invention, activated astrocytes may be used to reform or reinforce the blood brain barrier in patients in need of such treatment. Patients who may benefit from such treatment include but are not limited to patients in whom the integrity of at least a portion of the blood brain barrier has been disrupted by trauma, surgery, malignancy, infection, infarction, or exposure to a toxic agent, and thereby are susceptible to the deleterious effects of brain edema.

Such treatment may be especially useful following brain surgery or trauma, in which reforming or reinforcing the blood brain barrier may avoid post-trauma or post-surgery edema. Reforming or reinforcing the blood brain barrier should be construed to refer to the positioning of activated astrocytes along blood vessel walls to restore the relative impermeability of the blood brain barrier. In particular, the activated astrocytes may be applied to a wound perimeter from which they would then be able to migrate to appropriate positions around the blood vessels.

In a preferred embodiment of the invention, astrocytes for use in promoting nerve/blood vessel regeneration or reducing scar formation in a region of the CNS are initially derived from that region. For example, it is preferred to use activated astrocytes of the forebrain for treatment of a forebrain lesion, activated astrocytes of the spinal cord for treatment of a spinal cord lesion, and so on. However, astrocytes from regions other than the site of application can also be used.

Autologous transplants of activated astrocytes (i.e., the use of astrocytes derived from the same host) are preferred where possible. However, allogeneic and even xenogeneic astrocyte transplants may be used in the practice of the present invention and may, in fact, be more easily obtained and more practical to use in certain situations, such as emergency surgery. If cross-species transplants are used, closely related species are preferred. In cross-species transplants in which immunologic rejection is likely, such rejection may be reduced or prevented by means known in the art, i.e., immunosuppressive conditioning regimens including but not limited to chemotherapy (e.g., cyclophosphamide) and irradiation (see, e.g., Gluckman, E., et al., 1984, in Aplastic Anemia, Stem Cell Biology and Advances in

Treatment, Young, N.S., et al., eds., Alan R. Liss, Inc., New York, pp. 325-333) or by producing a barrier between transplanted cells and host tissue, for example by encapsulating the transplanted cells. Activated astrocytes may be provided using suitable cell lines.

5.2.2. PHARMACEUTICAL COMPOSITIONS

The present invention also provides for pharmaceutical compositions which comprise an effective amount of the activated astrocytes, and a pharmaceutically acceptable carrier. Such pharmaceutically acceptable carriers include sterile biocompatible pharmaceutical carriers, including, but not limited to, saline, buffered saline, dextrose, and other suitable aqueous solutions.

5.2.3. MODES OF DELIVERY

There is a wide range of methods which can be used to deliver the activated astrocytes of the invention for use in the promotion of CNS nerve or blood vessel regeneration and/or scar reduction.

In one embodiment of the invention, the activated astrocytes can be delivered by direct application, for example, direct injection of a sample of activated astrocytes into the site of nerve damage. In a particular embodiment, such astrocytes can be purified, for example, they can be cell line astrocytes or astrocytes purified from a tissue sample.

In a specific embodiment, the activated astrocytes can be delivered in a media which partially impedes their mobility so as to localize the astrocytes to a site of nerve damage. For example, activated astrocytes can be delivered in a paste or gel, e.g., a biodegradable gel-like polymer (e.g., fibrin, a hydrogel, etc.), or even contained in an embryonic tissue section placed at the site of nerve damage. Such a semi-solid media would also

have the advantage that it would impede the migration of (scar-producing) undesirable mesenchymal components such as fibroblasts into the site.

Other methods of using and applying activated astrocytes of the invention include but are not limited to the use of polymer implants and surgical bypass techniques. Such methods may be used together, alone, or in conjunction with other methods as described supra. Uses of polymer implants and surgical techniques are described in more detail infra.

5.2.3.1. THE USE OF POLYMER IMPLANTS

In a preferred embodiment of the invention, activated astrocytes can be applied to a site of nerve injury in a form in which the astrocytes are seeded or coated onto a polymer implant. An astrocyte-containing composition may be preferably applied to the implant in vitro, or alternatively, implants may be coated with activated astrocytes in vivo.

Various types of polymer implants can be used in this embodiment of the invention, with various compositions, pore sizes, and geometries. Such polymers include but are not limited to those made of nitrocellulose, polyanhydrides, and acrylic copolymers (see e.g., those described in European Patent Publication No. 286284, published October 12, 1988, by Valentini et al.; Aebischer, P., et al., 1988, Brain Res. 454:179-187; Aebischar, P., et al., 1988, Prog. Brain Res. 78:599-603; Winn, S.R., et al., 1989, Exp. Neurol. 105:244-250).

Various geometries of polymer implants are envisioned for use; in one embodiment, polymer implant shapes can be selected which tend to "mimic" the trajectory of the nerve pathway over which it is desired

to promote nerve regeneration. For example, as described infra in the examples sections, a pennant-shaped implant can be used to promote regeneration into the spinal cord dorsal root entry zone. Similarly, a "home-plate" shaped implant can be used in promoting nerve regeneration in the corpus callosum. Various other geometries are within the scope of the invention and may be used.

In another embodiment of the invention, polymers can be used as synthetic bridges, over which nerve regeneration can be promoted and scar formation can be reduced by application of activated immature astrocytes to the end(s), or in the vicinity of, the bridge. For example, an acrylic polymer tube with activated astrocytes at one or more ends, or throughout the tube, can be used to bridge lesions rostrally or bypass lesions, e.g., of the spinal cord, over which nerve regeneration can be induced. In a preferred embodiment, semi-permeable tubes may be used, e.g., in the dorsal columns or dorsal afferents, which tubes can contain and provide for the release of trophic factors or anti-inflammatory agents. The types of tubes which can be used include but are not limited to those described in various publications (see, e.g., European Patent Publication No. 286284, published October 12, 1988, by Valentini et al.; Aebischer, P. et al., 1988, Brain Res. 454:179-187; Aebischer, P., et al., 1988, Prog. Brain Res. 78:599-603; Winn, S.R., et al., 1989, Exp. Neurol. 105:244-250).

5.2.3.2. THE USE OF ACTIVATED ASTROCYTES IN CONJUNCTION WITH SURGICAL BYPASS TECHNIQUES

In another specific embodiment of the invention, the use of activated astrocytes is combined with surgical bypass techniques to promote nerve or blood vessel regeneration or to reduce scar formation in a given region.

In particular, surgical procedures which move a potentially regenerating fiber away from white matter and closer to its target grey matter can be used. Such procedures would tend to minimize the potential inhibitory influence of white matter (Caroni and Schwab, 1988, J. Cell Biol. 106:1281-1288; Caroni and Schwab, 1988, Neuron 1:85-96) on nerve regeneration. As one example, a cut dorsal root can be surgically implanted back into the spinal cord just lateral to its normal point of entry (i.e., the DREZ), so as to place the sensory fibers in closer proximity to their target, the grey matter of the dorsal horn, and minimize the inhibitory influence of nearby white matter structures such as the dorsal columns. The concurrent or subsequent provision of activated astrocytes (e.g., on a polymer implant) can then be used to induce axonal regeneration into the grey matter. Such a procedure is described by way of example in Section 11, infra.

In another embodiment of the invention, the use of activated astrocytes can be combined with surgical procedures for bridging and thus bypassing a CNS lesion. In one embodiment, the lesion can be a spinal cord lesion. If the spinal cord is completely injured at a particular level, then neural signals can neither ascend nor descend beyond this point. In an embodiment in which regeneration of the spinal sensory roots which enter the cord below the level of the lesion is desired, these roots may be cut and

rerouted to a new level of the spinal cord above the lesion. Since these roots must now travel a distance greater than they normally do, a "bridging" substrate for this additional growth is desired; such bridging substrates may be synthetic or natural, including but not limited to polymer tubes and autologous nerve grafts. For example, a nitrocellulose tube or an autologous peripheral nerve may be implanted to function as a bridge. Although regenerating nerves will readily grow on substrates such as polymer tubes or autologous nerve grafts, such nerves usually reenter the CNS only to a very limited extent. In order to promote their reentry into the CNS, activated astrocytes can be provided, in combination with such surgical techniques, in accordance with the present invention.

6. EXAMPLE: ACTIVATED IMMATURE ASTROCYTES PROMOTE NEURAL AND BLOOD VESSEL REGENERATION AND REDUCE SCAR FORMATION IN THE FOREBRAIN

6.1. MATERIALS AND METHODS

6.1.1. TRANSPLANTATION OF IMPLANTS AND CELLULAR COATINGS

6.1.1.1. INSERTION OF THE IMPLANTS

Timed pregnant C57 BL/6J mice were obtained from the Jackson Laboratories, Bar Harbor, Maine. The glial "sling", which forms the transient axon guidance pathway between the right and left cortical hemispheres of the brain, of 16 day embryos (E16) of timed pregnant mice (Silver, et al., supra 1982) was lesioned by inserting a microneedle into the embryos' calvarium approximately 1 mm rostral to the cranial landmark "lambda" to a depth of about 2 mm. As a result of the above process, the lesioned mice were consistently acallosal. In an experiment to test the efficiency of the technique, 50 animals were acallosal out of the 50 lesioned.

The lesioned embryos, once born, were then anesthetized and implanted with a specially designed piece of nitrocellulose filter (a 1-mm^2, "home-plate" shaped piece of cellulose membrane filter (Millipore), 0.45-μm pore size) on postnatal day (P) 2, 5, 8, 14, and 21 and at 8 months. In the neonates, the skull was still pliable and did not require drilling. An incision through the skin and cranium was made horizontally between the eyes and the skin was retracted. The surface of the skull was scraped free of tissue in order to minimize contamination of other cell types onto the implant as it was inserted. The specially designed implant was then inserted, pointed side first, 2-5 mm into the stab wound. The animals were then maintained in a normal growth environment for 2 or more days.

In addition, in order to determine whether a change in "activated" astrocyte morphology from stellate (i.e. inserted) to flat altered the efficiency of the astrocytes to provide a conducive substratum for axon elongation, a second set of experiments were conducted wherein portions of the implants were precrushed midsagitally to reduce pore size before insertion into the neonatal brain according to the above process.

6.1.1.2. REMOVAL OF THE IMPLANTS PLUS THEIR CELLULAR COATINGS

The implants, plus their cellular coatings, were removed from the decapitated acallosal mice 48 hours after implantation on postnatal day 2 (P2). The tissue around the implant was carefully dissected and the implant was removed with forceps which prevent the cells on the surface from being crushed or stripped away. The implants, and their cellular coatings, were then dipped in N-2 medium (Bottenstein, J.E. and Sato, G.H., 1983, Proc. Natl. Acad. Sci. U.S.A. 79:514-517) and placed in a humid chamber at 37°C until they were transplanted a few minutes later.

6.1.1.3. TRANSPLANTATION OF THE IMPLANTS AND THEIR CELLULAR COATINGS TO OLDER POSTCRITICAL PERIOD HOSTS

Host C57 BL/6J mice (at postnatal day 17, 34 or 8 months) obtained from the Jackson Laboratories, Bar Harbor, Maine, were made acallosal in the embryo or on the day of birth. The transplants (i.e. the implants plus their cellular coatings) were inserted in the same manner as described above.

The animals were then killed 0.5, 1, 2, 3, 5, 6, and 7 days and 2 months after implantation by perfusion through the heart. The perfusion was performed in two steps: first, 2-5 ml of an 0.15 M phosphate buffer solution at 37°C was injected into the left ventricle, followed by fixative (0.5% glutaraldehyde/2.0% formaldehyde in the same buffer, with 0.5% dimethyl sulfoxide (DMSO). The brains were quickly dissected from the cranium and placed in the same fixative overnight at 4°C. The filter and surrounding tissue were subsequently embedded in Spurr's plastic using standard procedures. Serial 1 μm sections were taken through the implant and stained with toluidine blue. Certain regions were sectioned ultra-thin, stained with uranyl acetate and lead citrate, and viewed with a Zeiss 109 electron microscope. For specimens examined by a scanning electron microscope (SEM), the tissue above the implant was gently dissected away and the specimens were osmicated and dehydrated through a graded series of alcohols. The samples were critical-point dried in a Balzers CPD 020 and sputtercoated with gold with an Edwards E306 device. After being mounted on aluminum stubs, they were viewed with an Etech scanning electron microscope.

6.1.2. IMMUNOHISTOCHEMISTRY

Variously aged postnatal C57 BL/6J mice made surgically acallosal and containing implants for one week were anesthetized and perfused through the heart with 2-5 ml of 4.0% formaldehyde in phosphate buffered saline (PBS, pH 7.5). The brains were dissected from their calvaria and immersed in fixative for 2 hours, then cryoprotected by using a graded series of sucrose PBS solutions (10% sucrose, PBS solution for 30 minutes, 15% for 30 minutes and 20% for 2 hours to overnight); 10-μm sections were taken on a Slee HR Mark II cryostat microtome.

Polyclonal antibodies against purified laminin and fibronectin were received from Dr. G. Martin (NIH). The sera were used at dilutions of 1:50. Antibodies against glial fibrillary acidic protein (GFAP) were provided by Dr. Robert Miller (Case Western Reserve, Cleveland, Ohio). They were diluted 1:1,000 and applied for 1 hour at room temperature. Sections were rinsed in PBS (3-15 minute washes) and incubated with peroxidase-conjugated goat anti-rabbit IgG (Cooper Biomedical, Malvern, PA) at a dilution of 1:100 for 30 minutes at room temperature or goat anti-rabbit FITC (fluorescein isothiocyanate) at a dilution of 1:50 for 1 hour. Sections were rinsed again in PBS, and peroxidase conjugates were incubated in a solution containing 15 mg DAB (3,3 diaminobenzidine tetrahydrochloride; Eastman Kodak Co., Rochester, NY) per 100 ml Tris (pH 7.5) for 30-45 minutes at room temperature in the dark.

6.1.3. HORSERADISH PEROXIDASE INJECTIONS

In order to determine the location of cell bodies that contribute axons to the implant surface, acallosal mice implanted on postnatal day 5 (P5) and allowed to survive 5 weeks were given a single, small wedge of crystalline horseradish peroxidase (Sigma VI) that was

inserted (with a spinal needle) very superficially into the cortex of one hemisphere of the brain in a region immediately lateral to the implant. On the following day, the animals were killed and perfused with 0.5% glutaraldehyde and 2% formaldehyde in 0.15 M PBS. Brains were removed and placed in the same fixative for 4 hours. Sections were cut on a vibratome at 65 μm and incubated in 50 mg of DAB in 100 ml Tris buffer (pH 7.5) for 20 minutes. A solution of 0.6% hydrogen peroxide was added and the sections were incubated 15-20 minutes longer. Sections were counterstained with neutral red and mounted on the slides.

6.1.4. ^{3}H-THYMIDINE AUTORADIOGRAPHY OF TRANSPLANTS

Acallosal neonates, implanted with filters on postnatal day 2 (P2), were injected intraperitoneally with ^{3}H-thymidine (5 μCi/g of body weight) at 6, 18, and 30 hours after implantation. Implants were removed 18 hours after the last injection and transplanted into postnatal day 23 (P23) surgically induced acallosal mice obtained from Jackson Laboratories. The mice were perfused 4 days after transplantation and prepared for plastic embedding. Sections 1 μm thick were mounted on slides and coated with Kodak NTB-2 autoradiographic emulsion. The coated slides were placed in light-proof containers and stored at -5°C for 6 weeks. Slides were processed for photography at 18°C.

6.2. RESULTS

6.2.1. DETERMINATION OF THE "CRITICAL PERIOD" IN ASTROCYTES FOR AXON ELONGATION

An analysis of the scanning electron micrographs of the acallosal mice implanted with untreated nitrocellulose bridges at various stages (P2, 5, 8, 14, and 21, and 8 months) indicated that the glial response at 24-

48 hours after implantation produced a terrain along the filter that was suitable for axon extension only in animals that were implanted before or on postnatal day 8 (P8). This was shown by the presence of many unmyelinated axons interspersed among the attached glial cells (astrocytes). See FIGURES 1 and 16. The micrographs indicated that glia in the P2 and P8 implants coated the majority of the implant surface, providing a substrate on to which axons and blood vessels were extended (FIGURES 4a through 4c). In P2 and P8 implants, the glia (astrocytes) on the filter appeared to be stellate in shape and respond to the presence of axons by sending out many cytoplasmic extensions around the fibers. See FIGURE 1d.

However, the CNS glial response generated by implantation of animals on or later than postnatal day 14 (P14) did not produce a terrain readily suitable for axon extension. The glia in the P14 animals appeared to be flat and lacked extensive infiltration of processes. The 27 animals given naked, untreated implants postnatally at 2 and 3 weeks and 8 months showed little or no growth of axons on to the implant when examined at 1 week and as late as 2 months after implantation (see FIGURE 2).

6.2.2. DETERMINATION OF THE LOCATION OF THE CELL BODIES THAT CONTRIBUTED AXONS TO THE IMPLANT SURFACE

A review of the micrographs concerning the horseradish peroxidase injections indicated that the representative sections contained retrogradely labeled cortical neurons in a position contralateral and homotopic to that of the injection site (see FIGURE 3). Thus, the results demonstrated that some of the commissured axons emerged from one region of the brain and grew across the midline to the opposite region of the brain, using the implant as a pathway.

6.2.3. HOST GLIAL RESPONSE TO NITROCELLULOSE BRIDGES IMPLANTED AT VARIOUS POSTNATAL AGES

Implantation of acallosal mice at various stages was done not only to evaluate the ability of the glial coating to provide an adequate substratum for axonal elongation but also to compare age-related changes in the host gliotic response. The micrographs showed that when implants were placed within the presumptive callosal pathway of acallosal mice at P2 and P8, the glial cells rapidly migrated onto the surface of the filter during the first 12-24 hours after implantation (See FIGURE 4). The glial cells attached themselves to the implant by extending their cytoplasmic processes deep into the implant's 0.45 μm pores.

The identification of the glial elements on the filter as astrocytes, as well as the extensive branching of their processes into both the prosthesis and encompassing tissue, were dramatically shown in the GFAP-stained sections (see FIGURE 5). In P2 implants there were only a few macrophages around the filter 48 hours after implantation and there was no evidence of tissue necrosis or persistent bleeding (see FIGURE 4).

In addition, the GFAP results demonstrated that the rapidity of astrocyte movement onto the filter and attachment with the filter decreased gradually as the age of implantation increased. In P8 implants examined after 48 hours, many GFAP-positive glia were already attached.

In contrast to P2 and P8 neonates, animals implanted on P14 and P21 showed only slight glial activity after implantation. Filters examined at this stage were coated mainly with degenerating tissue and vascular elements. Thus, the reaction and migration of glia onto the filter in animals implanted on or later than P14 required a longer period of time, often taking a full week for cells to reach the vicinity of the filter.

Moreover, the reactive older astrocytes had a conspicuous change in shape from stellate (i.e. inserted) to flat (compare FIGURE 2a with FIGURE 2e; also FIGURE 5a with FIGURE 5b). Macrophages with inclusions, mesenchymal tissues, and large amounts of necrotic debris always persisted within these developing scars. Another significant variation of the host glial reaction in older animals was the relative inability of the mature form of reactive astrocyte to insert processes into the implant. Filters introduced intracerebrally at later stages (P21 and 8 months), and examined after 7 days, had limited penetration of glial processes into their pores (FIGURE 2). Rather than inserting, the glia flattened on the surface of the filter and encapsulated the prosthesis by forming sheets several cell layers thick.

The anti-GFAP staining pattern at P21 showed sheets of flattened astrocytes having only a few short processes penetrating into the implant (FIGURE 5). These flattened astrocytes were often surrounded by nonstaining arachnoidal cells that composed a much larger proportion of the cell population encompassing the implant than at earlier stages.

6.2.4. EXTRACELLULAR MATRICES ASSOCIATED WITH GLIOTIC RESPONSE AT DIFFERENT AGES

The gliotic reaction that appeared 2-7 days after implantation in P2 neonates did not stimulate the production of collegen fibers or basal laminae within the parenchyma of the CNS. Only basal laminae that normally occur around capillaries and at the pial surface could be found. However, when animals implanted at P2 were examined for laminin (a major protein component of basal lamina), an unusual staining pattern was revealed. As expected, laminin appeared to be concentrated in the basal laminae of the blood vessels and the pia mater throughout the brain.

However, laminin was also found within the pores of the filter in regions containing inserted glial processes, sites having no observable amounts of basal lamina (compare FIGURES 6a, 6b with 6c, 6d), as well as in collagen associated with glial scar. Ectopic basal laminae first appeared in small, isolated patches among cells surrounding the implant in some P8 individuals examined after 2-7 days. Interestingly, in P8 animals, axons were not observed juxtaposed to the ectopic basal lamina. However, axons were observed clustered along the plasma membrane of astrocytes less than 10 μm away from the basal lamina (FIGURES 7b-d). The anti-laminin staining within the pores of the filter was greatly reduced or absent in brains implanted on or later than P14 (FIGURE 6d). Collagen fibers were seen throughout the scar, occupying spaces between cells and cell layers. Transmission electron microscope (TEM) examination of the banding pattern for the fibers identified them as being composed of type I collagen.

6.2.5. AXON REACTION TO FLATTENED ASTROCYTES IN POSTNATAL DAY 2 NEONATES INDUCED BY COMPRESSING THE PORES OF THE IMPLANT

Observation of the scanning electron micrographs taken 48 hours after the insertion of crushed implants on postnatal day 2, indicated that many of the GFAP-positive astrocytes in the crushed region spread out to become flat and often formed a confluent monolayer. In contrast, the astrocytes that accumulated in layers over the uncrushed region of the implant were stellate with many ruffles, blebs, and cytoplasmic extensions (see FIGURE 8).

In addition, the micrographs indicated that the growing axons extended over the "activated" stellate-shaped astrocytes infiltrating the porous portion of the implant but did not grow among the flattened astrocytes on the

crushed portion (see FIGURE 17). This experiment also demonstrated the importance of a proper pore size in the polymer for maintaining the astrocyte in an "activated" form when it is transplanted on nitrocellulose.

6.2.6. TRANSPLANTATION OF GLIAL-COATED IMPLANTS FROM NEONATAL TO POSTCRITICAL PERIOD ANIMALS

An analysis of coronal sections of postcritical period animals (i.e. P14 or greater) that received transplanted filters precoated with glia from neonates that were injected with ^{3}H-thymidine indicated that many of the inserted glia were indeed transferred and survived transplantation (see FIGURE 9). Silver grains were observed not only above glia attached to the filter but also above those along blood vessels which were well away from the surface of the implant. Thus, transplanted glia can migrate from the filter surface onto the blood vessels and may be able to reduce bleeding.

In addition, the micrographs indicated that the brains of animals receiving the transplants displayed distinct changes in glial reaction around the transplant when compared with those of the same age receiving untreated implants. Implantation of naked filters into P14 or older animals consistently resulted in rampant tissue degeneration, followed by the formation of a dense, flat-cell form of glial scar associated with extensive basal laminae and collagen fibers (see FIGURE 10).

In contrast, the majority of animals given transplants of activated immature glia (i.e. postnatal day 2 through 8) showed no scar formation, little basal lamina production, and negligible amounts of tissue necrosis and bleeding. In essence, the host gliotic response in transplanted animals became indistinguishable from animals implanted with naked implants during critical stages (FIGURES 10c, 10d). The transplanted animals also showed

an anti-laminin staining pattern identical to that seen in mice implanted on P2 (compare FIGURES 6a and 6b with FIGURES 6e and 6f). Most transplants examined 3-6 days after insertion showed (in regions where donor glia were present) little or no cellular debris and only a few macrophages at the donor/host interface (See FIGURES 10c, 10d, 11). The astrocytes along the surface of the implant formed multiple branches that interdigitated with the injured cortex, appearing to "knit" the artificial material with the tissue of the living host. In such animals (lateral to the longitudinal fissure), normal-appearing neuropil was present as close as one cell layer from the transplant (FIGURE 11). In these successfully transplanted animals, there was minimal invasion of arachnoidal cells into the wound site. However, in a few instances, dense collagenous scars with layers of basal lamina, fibroblasts, and flattened glia were located in discrete regions of tissue adjacent to areas of the transplant. This occurred primarily in those regions that lacked the penetrating, stellate form of astrocyte.

6.2.7. INDUCED AXON GROWTH OVER GLIAL TRANSPLANTS

In the coronal sections of a number of the postcritical period animals which had received implants with transferred glia, several hundred "regenerating" or sprouting axons were observed at the previously lesioned cerebral midline among the transplanted glia attached to the implant (see FIGURE 12). The axons were all unmyelinated (as expected for a newly regenerated axon) and bundled in small fascicles surrounded by glia.

6.3. DISCUSSION

A summary of the results produced in the above example is set forth below in Table I.

TABLE I.

Changes that Occur During and After Gliotic Reaction Induced When Accallosal Mice Are Implanted With Nitrocellulose Filters at Critical and Post-Critical Stages, and When Implants Are Transplanted From Neonates to Post-Critical-Period Animals[1]

	Critical		Post critical[2]	Transplant[3]
	P2	P8	P14-adult	P2 to P17,34
Number of inserted $GFAP^+$ cells	+++	++	-	++
Laminin along glial processes in filter	+	+	-	+
Axon outgrowth over filter	+++	++	-	+
Necrosis	-	+	+++	+
Blood surrounding filter	-	+	+++	+
Basal lamina	-	+	+++	+
Collagen	-	+	+++	+
Astrocyte shape	Stellate		Flat	Stellate
Inserted glial processes	+++	++	-	++
Time for glial to react to implant	24-48 hours		5-7 days	

1 The +- system represents an overall impression of the observablepresence or absence of the described reactions.
2 Untreated implants.
3 Implants from P2 critical period mice.

The present example demonstrates the existence of a "critical period" of less than postnatal day 8 for substrate-support axon regeneration. The de-novo growth of commissural axons across the cerebral midline was observed in acallosal animals implanted with untreated Millipore on or prior to postnatal day 8. In addition, the retrograde labeling studies using horseradish peroxidase showed that such fibers originated from cells of the cortex and terminated in the appropriate homotopic locations in the opposite hemisphere of the brain.

Moreover, in young mice (implanted before day 8) astrocytes did not produce a scar around a filter but instead sent many processes into the pores of the filter where the immature astrocytes produced a substrate which supported blood vessels and neurite growth. However, the results indicated that astrocytes in older mice (implanted on or later than postnatal day 14) failed to incorporate the filter within the brain and, instead, produced a glial-mesenchymal scar which did not support axon growth. The change in the brain's response to wounding, incorporation of implant, and support of axon growth indicated the presence of a "critical period", for substrate supported axon-elongation.

During the critical period, the migration of astrocytic glia onto the implant and the insertion of their processes are extremely rapid events occurring within 12-24 hours. The activated astrocytes were stellate-shaped and supported axonal elongation. During and after the initial glia invasion phase, the population of cells that moved onto the implant had the capacity to support the growth of axons, as well as vascular elements. The majority of these cells were $GFAP^+$ astrocytes and, while in their youth, they not only have the ability to erect a three-dimensional network of processes separated

by wide extracellular spaces, they also appear to respond to the presence of growing axons by extending additional cytoplasmic processes around the fibers.

In contrast, the results from the above example indicated that the reactive glial response in animals implanted 14 days or later after birth showed distinct differences from gliosis observed in neonates. The length of time it took glial cells to reach the surface of the implant site, the extent of secondary necrosis, the degree of basal lamina production and fibroblast contamination, and the density of tissue at and around the implant all increased with age (see Table I). The morphology of cells surrounding the implant also became altered (i.e. became flat as opposed to stellate in shape) and, most importantly, these cells lost their ability to stimulate axon outgrowth. Thus, in contrast to adult "reactive" gliosis (i.e. greater than postnatal day 14), the gliotic response in neonatal mammals (i.e. on or prior to postnatal day 8 in mice) is an active rather than reactive phenomenon and, when controlled geometrically with a prosthesis, can be considered a beneficial and constructive process.

As a result of the above discovery, activated immature astrocytes were transplanted into older animals to determine whether their "activated" effect could be transferred into the older animals to reduce the amount of tissue degeneration and glial scarring, as well as to determine whether the activated immature astrocytes could reestablish an environment conductive to axon regeneration. The results of the ^{3}H-thymidine tests indicated that when activated astrocytes were removed from a neonate (retaining structural integrity with a polymer prosthesis) and transferred to a more mature or adult acallosal animal, that most of the activated astrocytes were indistinguishable from "critical" stage mice

implanted with naked implants. Hence, the above results indicate that when activated immature astrocytes were implanted into an adult acallosal animal, an environment conducive for axon regeneration was reestablished in the host animal.

Moreover, the micrographs indicated that there was no evidence of tissue necrosis or persistent bleeding or scarring in the transplanted animal. The lack of extensive tissue degeneration and bleeding and scarring in the transplanted animals suggests that the transplanted astrocytes increased the survivability of cortical tissue near the site of the injury. Thus, the results demonstrate that the transplantation of "activated" immature astrocytes into post-critical period animals buffers the traumatic effect of the wound itself.

7. EXAMPLE: TRANSPLANTATION OF PURIFIED ACTIVATED IMMATURE ASTROCYTES INTO POSTCRITICAL PERIOD ANIMALS

7.1. MATERIALS AND METHODS

7.1.1. PREPARATION OF PURIFIED ACTIVATED IMMATURE ASTROCYTES

A highly purified population of "activated" murine immature astrocytes (before postnatal day 8 astrocytes) was prepared according to the following process. Cerebral cortices of four newborn C57 BL/6J mice obtained from the Jackson Laboratories were collected and cut into 1 mm pieces in 5 ml of calcium, magnesium-free Minimal Essential Medium (MEM-CMF). Trypsin (50% volume at 0.10%) was then added and the cells were incubated for 30 minutes at 37°C. Ethylenediamine-tetraacetic acid (EDTA) was then added (1 ml of 0.025% EDTA/5 ml of MEM-CMF) and incubated for 10 minutes. After incubation, the supernatant was then carefully removed and replaced with 2-3 ml of a mixture containing soybean trypsin inhibitor

(SBTI, 0.52 mg/ml), deoxyribonuclease (0.04 mg/ml) and bovine serum albumin (3 mg/ml) in Dulbecco's Modified Eagles Medium (DMEM). The tissue chunks were gently mixed and allowed to settle. The supernatant was removed and replaced by DMEM with 10% fetal calf serum (FCS). The tissue was triturated 5 times through a fire polished pasteur pipette. This was repeated once more with a second pipette that was fire polished a little longer so that the opening was approximately one third the diameter of the original pipette. The cell suspension was placed on ice (in a sealed 15 ml conical centrifuge tube) for 5 minutes and then centrifuged for approximately one minute at 100 rpm. The cell supernatant containing a suspension of single cells was transferred to another tube.

The cells were then counted and plated at a density of 2.0 to 10^7 cells/25 cm in a flask which had been previously coated with 0.1 mg/ml polylysine. The cells were pelleted by centrifugation at 1000 x g and suspended in 5 ml of 50% astrocyte conditioned medium (i.e., medium that had been taken from another 4 day culture containing astrocytes only) in DMEM containing 10% FCS. The majority of the astrocytes usually attached themselves to the culture plate within six to eight hours. Within this period of time, few neurons and non-astrocyte cells were attached to the dish. The non-astrocyte cells could easily be removed when the flask was shaken vigorously by hand. The cell suspension was then removed and fresh medium was added.

The cycle of shaking was repeated several times until only a few rounded cells appeared among the spreading cells. The cultures were shaken and refed once a day until all of the rounded cells were removed. This usually took three days. Hence, the resulting activated immature astrocytes obtained by this process were usually postnatal day 4 in development.

In order to obtain activated immature astrocytes which are embryonic, as opposed to postnatal in development, the above procedure was repeated utilizing embryonic day 18 (E18) fetal rat donors.

7.1.2. PREPARATION OF MATURE (P14 OR OLDER) ASTROCYTES

A portion of the above immature astrocytes were allowed to develop into mature astrocytes by the following procedure. Within 5-7 days after the initial plating, the astrocytes cultures became confluent. To replate the astrocytes, the media was removed, the cells were washed with MEM-CMF containing 0.02% EDTA and trypsin. This mixture was removed and only a few drops of this fresh medium was added back. The astrocytes were then incubated for 5-10 minutes until cells detached from the plates, suspended in DMEM and 10% FCS and transferred to a 76 cm culture flask. Two days after replating, the cultures were treated with a 2 day pulse of cytosine arabinoside (Ara-C) (2.5×10^{-5} M). The addition of Ara-C after each replating left a few dead cells, but controlled the proliferation of fibroblasts. Mature astrocytes were harvested from cultures 28 days or older.

7.1.3. SEEDING OF THE NITROCELLULOSE IMPLANTS

The astrocytes produced above were removed from their cultures using MEM-CMF containing 0.02% EDTA and trypsin. After approximately 10 minutes, cold DMEM with SBTI and deoxyribonuclease (DNase) were added to the cell suspension. The cell suspension was then pelleted by centrifugation at 1000 g for five minutes. The supernatant was removed and the astrocytes were resuspended in 1 ml DMEM. The astrocytes were then pelleted and resuspended in DMEM a total of three times,

the last in only 200 μl Cell viability and number were then determined by using trypan blue exclusion, which showed that many cells survive the procedure.

The implants to be inserted into the forebrains and spinal cords of the host animals were prepared out of various pore sizes of nitrocellulose filter paper. The implants to be inserted into the forebrain of postnatal day 60 (P60) acallosal mice were prepared out of 0.45 μm pore size nitrocellulose filter paper and were of the same "home plate" shape and size as the implants defined in Example 1 (i.e. 1-mm^2). The implants to be inserted into the dorsal root entry zone (DREZ) of the spinal cords of the root-lesioned adult rats were prepared out of 8.0 μm pore size nitrocellulose filter paper and were "pennant" shaped and of a size approximately 0.75-1.0 mm x 3.0 mm x 250 μm. See FIGURE 18.

Approximately 10 ml of the activated immature astrocytes were then seeded onto the nitrocellulose implants at a density of 10^7 cells/ml (about 10^5 astrocytes/implant). The astrocytes suspension usually formed a bead on the surface of the filter, and was incubated for two hours at 37°C. A 100 μl drop of DMEM was then carefully placed over the nitrocellulose implant and the astrocytes were allowed to attach themselves to the filter by incubation overnight.

The above seeding process was then repeated to produce the comparison implants containing the mature mouse astrocytes. However, mature (P14 or older) mouse astrocytes were substituted for the activated immature astrocytes.

7.1.4. IMPLANTATION OF THE GLIAL-COATED FILTERS

7.1.4.1. IN THE FOREBRAIN OF THE POSTNATAL DAY 60 ACALLOSAL MICE

The forebrain "home plate" shaped implants coated with the activated immature astrocytes were inserted into eight postnatal day 60 acallosal mice according to the procedure set forth in Example 1. For comparison purposes, "home plate" shaped implants coated with mature (P14 or older) implants were also implanted in a similar number of postnatal day 60 acallosal mice by the same procedure utilized above.

7.1.4.2. IN THE DORSAL ROOT ENTRY ZONE OF THE SPINAL CORD OF THE POSTNATAL DAY 180 OR OLDER RATS

Postnatal day 180 or older (200-250 gram) rats obtained from Charles River Facilities were anesthetized. A laminectomy was performed over dorsal roots L4-L6 to expose them and their entrance points into the cord. Dorsal roots L4 and L6 were cut as a means of control to localize the sensory perception of dorsal root L5. Dorsal root L5 was subsequently crushed (3X for 10 seconds). Dorsal root L5 was then traced to the dorsal root entry zone in the spinal cord where a 1.5-2 mm superficial incision (approximately 0.5 or less mm in depth) was made into the spinal cord between the dorsal root (L5) and posterior columns. The pennant shaped implant coated with activated immature rat astrocytes was then inserted superficially into the incision. See FIGURE 18. The pole portion of the pennant-shaped implant was pushed gently into the root. Thus, while the broad portion of the pennant laid in the cord proper, just medial to the dorsal horn, the pole portion of the pennant-shaped implant protruded gradually outside the spinal cord and into the dorsal root itself.

The incision and pennant were then covered with sterile gelfilm and the muscles and skin were sutured to cover the wound. At 2 day intervals for three weeks to one month the rats were tested to see if they had recovered any of their L5 dorsal root sensory motor behavior. In addition, after 21-30 days, the rats were sacrificed and serial sections of the implant and surrounding tissue region were prepared for examination according to the procedure set forth in Example 1.

7.1.5. IMMUNOHISTOCHEMISTRY AND ^{3}H-THYMIDINE AUTORADIOGRAPHY OF THE TRANSPLANTS

The same procedures set forth in Example 1 were applied to the immature and mature astrocytes of the present examples.

7.2. RESULTS

7.2.1. TRANSPLANTATION OF PURIFIED ASTROCYTES INTO THE FOREBRAIN

The transplantation of activated immature purified astrocytes into the forebrain of postnatal day 60 (P60) mice repressed scar formation in five out of the eight mice tested. These transplanted animals resembled those receiving non-glial coated implants on postnatal day 8 (P8). Scar formation was repressed over the majority of the implant surface. In addition, the immunocytochemical staining of immature astrocytes transplanted into postnatal day 60 (P60) mice indicated that the cells attached to the implants were GFAP-positive and many had processes deep within the pores of the filter. The ^{3}H-thymidine autoradiography results of the transplants showed that after seven days following astrocyte insertion into the brain, labeled cells often were found away from the implant surface (see FIGURE 13).

To determine the amount of scar surrounding the transplants, sections consecutive to that in FIGURE 13a were stained with antibodies against laminin protein. On the dorsal portion of the implant, which was the side coated with astrocytes from culture, the laminin staining pattern was confined to the filter surrounding astrocyte processes and to the basal lamina surrounding blood vessels (see FIGURE 13c). This staining pattern was similar to that observed with neonatal-implants and the transplants of Example 1. At the bottom (ventral part) of the filter, basal lamina within the scar was apparent. This area did not show GFAP-positive astrocyte processes within the implant. Since astrocytes were only plated on the top portion of the filter, scar formation at the bottom of the filter was a consistent feature of all transplanted adults receiving implants. The uncoated bottom portion of the filter also served as an internal control.

When astrocytes were allowed to mature in culture for 28 days, their relative size greatly increased and their rate of division decreased. Shortly after replating, mature astrocytes hypertrophy, in which the astrocytes spread to sizes between 50 to 120 μm.

In contrast to the immature astrocyte transplants, transplantation of mature astrocytes on filters (28 days or more in culture) failed to repress scar formation (see FIGURE 14). In such animals, thick dense scars covered the majority of the implant surface (see Table II below and FIGURE 14). Electron micrographs of the transplant showed that the scar was similar in morphology to that which occurred when untreated filters were implanted into adult forebrains. The scar was predominantly composed of fibroblasts and astrocytes. Extracellular matrix components such as basal lamina and collagen were also present (FIGURE 15b). Importantly,

mature astrocytes labeled with ^{3}H-thymidine prior to transplantation did not migrate out of the scar and many remained attached to the implant (see FIGURE 13f). This was remarkably different from transplants of immature astrocytes (compare FIGURE 13c to FIGURE 13f).

TABLE II

		Area of Implantation	
Age		Scar Formation	Glial Incorporation
P 2-48 hr	n=20	2.15 +/- 0.78	38.6 +/- 9.07
P 8-48 hr	n=10	8.2 +/- 2.71	30.6 +/- 9.75
P14-7 days	n=6	26.8 +/- 11.07	17.0 +/- 7.02
P21-7 days	n=16	35.75 +/- 9.02	5.3 +/- 1.70
Trans P2 to P34	n=15	5.4 +/- 1.81	35.5 +/- 8.94
In vitro 2 days to P60	n=8	12.5 +/- 1.19	38.0 +/- 0.75
In vitro 28 days to P60	n=10	43.6 +/- 4.9	5.2 +/- 5.0

This table quantitatively illustrates the amount of scar which forms on the surface of the implant at different ages or conditions. Scar formation was quantified by dividing the dorsal portion of the filter surface into numerous grids (each approximately 50 μm). Each grid was scored and counted for scar formation or the ability of the glia to incorporate the implant into the host brain according to definitions described above. The amount of scar formed after implantation into P2, 8, or

P34 transplants is greatly reduced when compared to animals implanted on P14 or older. Difference between acallosal mice implanted on P21 and those implanted on P2, 8, or transplanted is significant to P less than 0.01.

7.2.2. TRANSPLANTATION OF PURIFIED ACTIVATED IMMATURE ASTROCYTES INTO THE DORSAL ROOT OF THE SPINAL CORD

The micrographs obtained of the pennant-shaped nitrocellulose implant coated with purified activated immature rat astrocytes inserted in the dorsal root of the spinal cord indicate that the combination of embryonic astrocytes plus an oriented nitrocellulose implant represses scar formation locally in the spinal cord dorsal root entry zone (DREZ) and stimulates axons and blood vessels to enter the central nervous system along the implant surface. (See FIGURES 19a through 19c). In addition, the horseradish peroxidase (HRP) labelling studies of the previously lesioned L5 root show many fibers and terminals with buttons in their proper positions of laminal 2 and 3 within the dorsal horn. The exact relationship of the incoming fibers with the implant surface and the presence of a number of novel terminal arbor malformations in a subpopulation of the axons provides convincing evidence that the fibers in the cord are truly regenerated.

Moreover, six (6) of the nineteen (19) rats implanted with the pennant-shaped nitrocellulose implants coated with activated immature rat astrocytes exhibited functional recovery of many of their basic sensory motor behaviors approximately 5 to 7 days following insertion of the implants. This functional recovery was shown to be due, at least in part, specifically to the promotion of nerve regeneration, as demonstrated by the loss of functional recovery upon relesion of the nerve growing

along the nitrocellulose implant. Hence, the above results demonstrate that activated immature astrocytes seeded onto nitrocellulose implants promote directed axonal and blood vessel regeneration and repress glial scar formation in the spinal cord.

8. EXAMPLE: IMMORTALIZATION OF THE ACTIVATED IMMATURE ASTROCYTES

Purified activated immature (postnatal day 0) rat cortical astrocytes produced according to the process set forth in Example 2 were immortalized with defective retrovirus coding for SV40 T antigen and bacterial neomycin resistance genes by the following procedure. The purified activated immature astrocytes were seeded at about 5 x 10^5 cells per 60 mm dish on day 1. On day 2 the medium was replaced with 2 ml of the viral supernatant containing the defective retrovirus coding for SV40 T antigen and bacterial neomycin resistance genes (obtained from Dr. P.S. Jat, University College at London, London, England). The cells were incubated for 2-3 hours and then the virus was removed and fresh medium was resupplied. Neomycin resistant colonies were selected with medium containing G418 (a neomycin analog obtained from Gibco, Grand Island, N.Y.). Colonies which displayed astrocyte morphology were selected and cultured over a period of months, and were passaged without undergoing microscopically observable change. The clones were then stained for glial fibrillary protein and tested for promotion of axonal growth. The clones were GFAP-positive and promoted axonal growth to a similar extent as immature primary rat astrocytes, and thus the activated immature phenotype was preserved.

9. EXAMPLE: ASTROCYTE MATURATION REDUCES NEURITE OUTGROWTH AND NEURONAL ADHESION IN VITRO

We have examined neurite outgrowth on, and neuronal cell adhesion to, purified populations of immature and mature astrocytes in vitro. We show that the rate of neurite outgrowth is consistently reduced over the surface of mature astrocytes compared with immature astrocytes, and in short term adhesion assays, neuronal cell body adhesion is dramatically reduced on mature compared to immature astrocytes. These effects appear to be mediated through cell-surface changes in the astrocytes.

9.1. MATERIALS AND METHODS

9.1.1. PREPARATION OF PURIFIED ASTROCYTES

Purified astrocytes were prepared from new born rat cerebral cortices using a modification of the method described by Cohen (1983, Handbook of Laboratory Methods, University College, London). Briefly, cerebral cortices were carefully dissected from newborn mice, the meningeal tissue stripped off, and cells dissociated by incubation in 0.025% trypsin in calcium-magnesium free buffer (MEM-CMF) for 30 minutes. Cells were pelleted at 1000 x g for 5 minutes, resuspended in 5 ml of 50% astrocyte conditioned medium in DMEM containing 10% FCS and plated in polylysine coated (0.1 mg/ml) 75 cm^2 tissue culture flasks at a density of 2.01×10^7 cells/flask and incubated at 37°C with 5% CO_2 for 4-6 hours. Non-adherant cells were removed by shaking and the media was changed. Greater than 95% of the cells that remain attached to the flask following this procedure were astrocytes as defined by staining with antibodies to GFAP, and although these cells assumed various morphologies, greater than 95% had an antigenic phenotype similar to type-1 optic nerve

astrocytes (Raff, M.C., et al., 1983, J. Neurosci. 3:1289-1300). Immature astrocytes were harvested after two further days in culture, while mature astrocytes were harvested after a further 28 days in culture. Cells were removed from tissue culture flasks by incubation in 0.025% EDTA and 0.025% trypsin in MEM-CMF for 15 minutes. They were pelleted at 1000 x g for 5 minutes, resuspended in DMEM-F12, 10% FCS to such a concentration that 25 μl of medium contained 60,000 astrocytes, and plated onto a central region of polylysine coated glass coverslips to form confluent monolayers.

9.1.2. PREPARATION OF CORTICAL NEURONS

Highly enriched populations of embryonic cerebral cortical neurons were prepared from embryonic day 18 (E18) rats. The cerebral cortices were dissected, meninges removed, and the tissue cut into approximately 1 mm^3 pieces. Tissue was incubated in calcium-magnesium free buffer (CMF) containing 0.01% trypsin and 0.025% EDTA for 30 minutes, an equal volume of DMEM containing 10% FCS and 0.05 mg/ml DNase was added and the incubation continued for a further 5 minutes. Cells were dissociated by trituration, pelleted at 1000 x g and resuspended in DMEM-F12 with 10% FCS. Debris and non-dissociated cells were removed by passage through a 30 μm nylon filter and the resultant cell population plated at a density of approximately 2.5 x 10^6 cells/25 cm^2 poly-l-lysine coated flask and incubated for 4 hours at 37°C in 5% CO_2. The media was then replaced with 3 ml of 0.025% EDTA in MEM-CMF (Spinners) and incubated for a further 20 minutes. Neurons were detached from the substrate by gentle shaking and removed from the flask with the medium. After chilling on ice, cells were pelleted and resuspended in DMEM-F12 containing 1.0% FCS and 2 mM sodium pyruvate.

9.1.3. QUANTITATION OF NEURITE OUTGROWTH

To quantify neurite outgrowth, 1 ml of media containing 5000 neurons form E18 cerebral cortices was plated onto the surface of an astrocyte monolayer or a non-cellular substrate (laminin, astrocyte conditioned media etc.). The extent of outgrowth was assayed 16, 28 and 40 hours after plating as described below.

Neurons were identified by labelling with the C fragment of tetanus toxin conjugated to texas red (a gift from Dr. N. Robbins). Cultures were incubated for 30 minutes at room temperature in a 1:20 dilution of the tetanus toxin conjugate in phosphate buffer in the presence of 50% normal goat serum (NGS) or 5% BSA. After washing, cultures were fixed with 4% paraformaldehyde and mounted in 5% n-propyl gallate in glycerol. For double labelling experiments, following fixation, cultures were permeablized with acid-alcohol at -20°C and incubated in anti-GFAP serum (Pruss, R.M., 1979, Nature 280:688-690) at a dilution of 1:1000 in PBS and 10% NGS for 1 hour at room temperature, washed, and incubated in goat anti-rabbit Ig conjugated to fluorescein (G RIg-FITC) (Capel) at a dilution of 1:100 in PBS and 10% NGS for 1 hour at room temperature. Cultures were washed, mounted and examined with a Nikon optiphot photomicroscope equipped with epifluorescence.

Individual neurites grown for 28 hours on substrates of either poly-l-lysine (100 μg/ml), laminin (25 μg/ml), immature or mature astrocyte conditioned media, and a combination of laminin and astrocyte conditioned media were also photographed for measurement. Astrocyte conditioned media was obtained by incubating confluent monolayers of astrocytes in DMEM-F12 without serum for 48 hours prior to addition to coverslips. Polylysine coated coverslips were treated with laminin and/or astrocyte conditioned media for 8 hours or

overnight prior to the addition of neurons. Neurons grown on astrocyte conditioned media treated coverslips were also plated in the presence of 50% astrocyte conditioned media.

Photomicrographs of individual neurons with clearly identifiable neurites were taken using a 20x fluoro/phase lens on tri-X film rated at 400 ASA. The 35 mm negatives were projected at a fixed magnification onto the screen of a Vanguard Motion Analyzer and lengths of individual neurites measured using a Numonics digitizer. For each time point, 30-40 neurites were measured over both immature and mature astrocytes in three separate experiments. The data was pooled and the mean of the longest neurites was calculated. Comparison of neurite lengths was made using standard student T-test.

9.1.4. SHORT TERM NEURONAL ADHESION ASSAY

To compare the adhesive properties of immature and mature monolayers of astrocytes for cortical neurons, E18 cortical neurons were labelled with fluorescein diacetate (Rutishauser, U., et al., 1976, Proc. Natl. Acad. Sci. U.S.A. 73:577-581). Neurons dissociated from E18 rat cerebral cortices were incubated in 200 μg of fluorescein diacetate in 10 ml DMEM at room temperature for 15 minutes. Labelled cells were then pelleted at 1000 x g for 5 minutes, washed, and resuspended to a concentration of 5 x 10^5 neurons/ml in DMEM. One ml of cell suspension was added to an individual well in a 24 well plate containing a coverslip coated with a monolayer of either immature or mature astrocytes, incubated for 30 minutes on a rotating shaker at 75 RPM at room temperature and gently washed to remove non-adherent neurons. Neuronal adhesion was assayed by counting the

number of attached labelled neurons in 30 consecutive randomly selected fields under fluorescein optics using a 20X lens.

9.1.5. ELECTRON MICROSCOPY

Cultures of young and old astrocytes, removed at the time of plating neurons, were fixed by immersion in a fixative containing 3% paraformaldehyde, 1% glutaraldehyde in 0.12 M phosphate buffer. Cells were fixed for 3 hours at room temperature, washed in buffer and post fixed in 1% osmium tetroxide for 1 hour on ice. After washing, cultures were en-block stained with 0.5% aqueous uranyl acetate for at least 4 hours at 4°C, washed, dehydrated through graded alcohols and embedded in Epon 812 resin. After removal of the glass coverslip, en-face sections were cut on an LKB microtome, stained with uranyl acetate and lead citrate and examined on a JEOL 100 CX electron microscope at 80KV.

9.2. RESULTS

9.2.1. NEURONAL MORPHOLOGY ON IMMATURE AND MATURE ASTROCYTES

Embryonic neurons grown on immature and mature astrocytes assumed a variety of different morphologies. They could be broadly categorized into three distinct classes. Approximately 30% of the neurons extended multiple neurites of similar length and lacked any long axon-like projection (Fig. 20A). Neurons with this morphology may represent interneurons in the intact CNS. Approximately 40% of the neurons extended many short neurites and a single long neurite 2-3 times the length of the other neurites (Fig. 20B). Neurons with this morphology may represent short projection neurons in the intact CNS. Approximately 25% of the neurons extended

many short neurites and a single very long neurite often with an identifiable growth cone (Fig. 20C) and may represent long projection neurons in the intact CNS.

To determine if these different cell morphologies represented distinct populations of neurons, or a maturation of cells extending multiple short neurites to those which form long projection neurites, we examined the number and morphologies of the neurons that attached to young and old astrocyte monolayers at different times after plating. We found that the relative numbers in each class of neurons were similar on both young and old astrocytes (Table III), and in both cases the number of neurons that survived and sent out processes was very similar (Table III).

TABLE III

RELATIVE NUMBER OF EACH TYPE OF NEURON ON IMMATURE AND MATURE ASTROCYTES AFTER 28 HOURS*

Neuron type	Immature (P1-3d) Astrocytes	Mature (P1-34d) Astrocytes
Interneuron (a)	34.25+/-3.11	34.25+/-2.56
Short Projection (b)	38.00+/-2.93	38.12+/-2.36
Long Projection (c)	23.75+/-4.11	23.12+/-1.13
Total	96.00+/-9.26	95.50+/-5.00

* Neurons plated on the surface of astrocyte monolayers were cultured for 28 hours, labelled with tetanus toxin and examined as described in methods. Twelve randomly selected fields were classified on their morphology as shown in Figure 20. Note that the survival of the different populations of neurons is very similar on both immature and mature astocytes.

Although with longer culture periods the length of the longest neurites increased, the ratio of the number of neurons with different morphologies was nearly identical at 16, 28 and 40 hours after plating. These observations suggest that the three classes of morphologically distinct neurons seen in these cultures represent distinct populations of CNS neurons, and that the number and relative proportions of neurons that survived was similar on both young and old astrocytes.

9.2.2. DIFFERENCES IN NEURITE OUTGROWTH OVER IMMATURE AND MATURE ASTROCYTES

Comparison of neurite outgrowth over the surface of young and old astrocyte monolayers 16 hours after plating showed that neurites growing over the surface of immature astrocytes appeared longer than the neurites from a matched population of cells growing over the surface of mature astrocytes (Fig. 21; compare a and b). This difference was also apparent when neurons were grown for 28 or 40 hours over either immature or mature astrocytes. To determine more precisely the difference in neurite length on the two glial substrates, the length of the longest neurites were measured for 100 neurons at each time point.

At all three time points, neurons growing on the surface of the young astrocytes extended neurites that were approximately 30% longer than those neurites extending from neurons growing on the surface of old astrocytes (Table IV).

TABLE IV

NEURITE OUTGROWTH OVER IMMATURE AND MATURE ASTROCYTES*

Astrocyte Type	Outgrowth Time (hours)	Length of Neurite Outgrowth (!)		
		Longest	+Branches	Total
Immature	16	215.1+/-26	252.1+/-33	316.5+/-63
	28	372.6+/-38	421.9+/-48	527.5+/-78
	40	490.5+/-53	549.4+/-60	702.8+/-102
Matrure	16	141.1+/-17	167.1+/-22	231.5+/-46
	28	263.0+/-26	298.7+/-32	390.5+/-56
	40	341.1+/-37	394.6+/-42	556.2+/-82

* The length of the longest neurite, neurite and associated branches and total neurite outgrowth was measured from 100 neurons at three different times after plating on monolayers of immature and mature astrocytes. Note that in all cases the outgrowth over the surface of immature astrocytes was significantly greater than over the surface of mature astrocytes. Significance of difference in outgrowth was determined by standard student T-test. $P < 0.001$ for length of longest neurite and longest neurite with associated branches, $P < 0.01$ for total outgrowth.

The rate of neurite extension over the glial surfaces appeared relatively linear over the time course of the experiment (Fig. 22), although it was always more rapid over the surface of young astrocytes than over the surface of old astrocytes.

To determine if this apparent more rapid rate of neurite extension on the surface of the young astrocytes reflected a decrease in neurite branching, the total length of the longest neurite and its associated branching was measured. Again, a 30% increase in outgrowth was found on the surface of young astrocyte monolayers compared to old astrocyte monolayers (Table IV). To ensure that this increase in the length of the longest neurite represented an increase in neurite production and not a redistribution of neurite extension, the total neurite outgrowth from neurons on the surface of immature and mature astrocyte monolayers was compared. Total neurite outgrowth was more than 25% greater from neurons on the surface of immature astrocytes than it was from neurons on the surface of mature astrocytes, suggesting that those neurons associated with immature astrocytes synthesized comparatively more neurites than those neurons on the surface of mature astrocytes.

9.2.3. NEURITE OUTGROWTH IN THE PRESENCE OF CONDITIONED MEDIUM FROM IMMATURE AND MATURE ASTROCYTES

To determine if the difference in neurite outgrowth over the surface of immature and mature astrocytes resulted from the release of different soluble factors by the astrocyte monolayers or was mediated directly through interactions with the astrocyte surface, neurite outgrowth was examined in the presence of immature and mature astrocyte conditioned media.

Comparison of neurite outgrowth over a poly-l-lysine substrate alone, or poly-l-lysine treated with either immature, mature astrocyte conditioned media, or a combination of laminin and either immature or mature astrocyte conditioned medium indicated that both young and old astrocyte conditioned media resulted in a similar increase in axonal growth over that seen on a polylysine substrate. The extent of this neurite outgrowth was comparable to that seen over the surface of laminin coated polylysine. The addition of astrocyte conditioned media to laminin coated coverslips did not result in any additional increase in axonal outgrowth (Table V).

TABLE V

NEURITE OUTGROWTH ON NON-CELLULAR SUBSTRATES*

Substrate		Length of Longest Neurite (μm)
Poly-l-lysine	n=57	54.4+/-21.2
Laminin	n=51	153.2+/-26.9
Immature Astrocyte Condition Media	n=51	144.7+/-30.5
Mature Astrocyte Condition Media	n=54	138.0+/-32.8
Laminin + Immature Astrocyte Condition Media	n=52	159.6+/-34.3
Laminin + Mature Astrocyte Condition Media	n=51	152.0+/-34.1

* Neurons plated on non-cellular substrates were measured after 28 hours in culture as described in methods. All coverslips were coated with 100 μg/ml polylysine. Laminin substrates were produced by coating with 50 μl of a laminin (25 μg/ml) for 8 hours. Media (DMEM-F12) was conditioned by confluent immature and mature astrocyte cultures for 48 hours prior to adding 50 μl to either poly-lysine or laminin coated coverslips. Note that laminin and conditioned media from both immature and mature astrocytes result in a similar increase in neurite outgrowth over a polylysine substrate, but these increases do not appear to be additive.

In all cases, neurite outgrowth was >40% less on laminin and astrocyte conditioned media than over the surface of mature astrocyte monolayers (compare Tables IV and V). However, significant differences in neuronal morphology were seen on these substrates. On laminin, the majority of neurons extended few neurites, usually having only a single unbranched process, while in the presence of both young and old astrocyte conditioned media, although there appeared to be an increase in the number of neurites, this was always less than that observed from neurons grown on astrocyte monolayers.

9.2.4. NEURON ADHESION TO IMMATURE AND MATURE ASTROCYTES

To compare the adhesion of cortical neurons to monolayers of either immature or mature astrocytes, the relative number of attached neurons was determined following a short term adhesion assay. Neurons preferentially attached to the astrocyte monolayers, rather than the surrounding poly-l-lysine coated coverslip. The extent of neuronal adhesion to the surface of immature astrocytes was considerably greater than neuronal adhesion to the surface of mature astrocytes (Fig. 23). Quantitation of this adhesion showed an approximate 4 fold increase in the number of neurons binding to the surface of immature compared to mature astrocytes (Table VI).

TABLE VI

NEURON ADHESION TO ASTROCYTE MONOLAYERS*

Astrocyte	Number of Neurons
Immature	149+/-36
Mature	39+/-13

* Neurons at a density of 5×10^5 were plated on the surface of monolayers of immature and mature astrocytes for 30 minutes, and non-adherent cells were removed. Twelve random fields were counted from 3 different preparations as described in methods, and the data pooled. Note that there is a greater than three fold increase in the adhesion of neurons to immature astrocytes compared to mature astrocytes. Significance of difference using student T-test $P<0.001$.

9.2.5. ULTRASTRUCTURAL CHARACTERISTICS OF IMMATURE AND MATURE ASTROCYTES

Maturation of forebrain astrocytes appears to cause a reduction in neurite outgrowth and neuron adhesion. This reduction may result from a reduction in viability or increase in cell death in the astrocyte cultures over time, or the replacement of the immature astrocyte phenotype by a mature astrocyte phenotype. To assess these possibilities, confluent monolayers of both immature and mature astrocytes were examined by transmission electron microscopy. Neither cultures exhibited signs of necrosis, or extensive cell damage, although the cells in both cultures expressed many

characteristics of astrocytes in vivo (Fig. 24). However, morphological differences between the cells in the two cultures were evident. Immature astrocytes had a dense cytoplasm containing many closely packed organelles, few dense bodies and small numbers of intermediate filaments (Fig. 24a, b). The immature astrocytes were usually small, with few junctional specializations and large spaces between them filled with many thin cytoplasmic processes (Fig. 24a). By contrast, mature astrocytes had a less dense cytoplasm, containing less closely packed organelles, but many dense bodies and large numbers of intermediate filaments (Fig. 24c, d). The mature astrocytes were large cells and appeared to be in closer apposition with one another and contained many more junctional specializations between them (Fig. 24c). Thus, ultrastructurally it appears that the reduction in neurite outgrowth seen over the surface of mature astrocytes may reflect the development of a mature astrocyte phenotype and not a reduction in viability or increase in cell death of astrocytes during the culture period.

9.3. DISCUSSION

We have previously shown that an enriched population of immature astrocytes transplanted into the forebrain of acallosal adult mammals have the capacity to suppress glial scar formation and support axonal growth, and that this capacity to support axonal growth appeared to be lost upon maturation of the astrocytes (see Sections 6 and 7, supra). Here we show that in vitro maturation of a purified population of astrocytes results in a reduction in both the capacity of these cells to support axon outgrowth and in their adhesive properties to neuronal cell bodies. These results provide direct

evidence that maturation of astrocytes results in a reduction in their capacity to support neurite outgrowth and their adhesive properties for neurons.

From both morphological and functional analyses, the maturation of astrocytes appears to occur on a similar time schedule both in vivo and in vitro. These studies help identify what has been termed a "critical period" which ends around postnatal day 8 in rodents. This critical period was also apparent in cultured astrocytes, so that astrocytes removed from newborn mice brains and maintained in culture less than 7 days suppressed glial-scar formation and supported axonal growth when transplanted into an adult brain (Smith, G.M., and Silver, J., 1988, in Progress in Brain Research, Vol. 78, Ch. 45, Gash, O.M. and Sladek, J.R., Jr. (eds.), Elsevier Science Publishers). On the contrary, astrocytes removed from newborn brain and cultured for several weeks failed to support axon growth when transplanted into the adult brain (Smith, G.M., and Silver, J., 1988, in Progress in Brain Research, Vol. 78, Ch. 45, Gash, O.M. and Sladek, J.R., Jr. (eds.), Elsevier Science Publishers).

Given that astrocyte maturation in vivo and in vitro appears to result in similar morphological phenotypes, it was surprising that there was only a 30% reduction in the extent of neurite growth over the surface of mature compared to immature astrocytes in culture, since very limited axon growth is seen following a lesion to the adult CNS (Kiernan, J.A., 1979, Biol. Rev. 54:155-197). A number of factors may contribute to this apparent discrepancy. Recent studies have suggested that specific proteins found on the surface of oligodendrocytes and in myelin of the mammalian CNS but not PNS have the capacity to strongly inhibit axon outgrowth (Caroni, P., and Schwab, M.E., 1988, J. Cell

Biol. 106:1281-1288; Schwab, M.E., and Caroni, P., 1988, J. Neurosci. 8:2381-2393). Given that myelin debris is generally present after lesions to the adult CNS, it would contribute to the inhibition of axon growth, while such myelin debris is absent from the purified astrocyte cultures. Furthermore, lesions to the adult CNS usually result in a gliotic response or gliosis. In vivo, gliosis consists of what has been termed "reactive" astrocytes which contain an abundance of intermediate filaments and intracellular junctions (Reier, P.J., et al., 1983, in Spinal Cord Reconstruction, Kao, C.C., et al. (eds.), Raven Press, New York, pp. 163-195; Nathaniel, E.J.H. and Nathaniel, D.R., 1981, in Advances in Cellular Neurobiology, Vol. 2, Academic Press, New York, pp. 249-301). Although mature cultured astrocytes also contain an abundance of intermediate filaments, intracellular junctions and have a large cytoplasmic volume, their relationship to "reactive" astrocytes in vivo is unclear. Thus, it is conceivable that a particular population of mature astrocytes in vivo may respond directly to a lesion and change in such a way as to totally inhibit axon growth. Another possible explanation for inhibition of axon regeneration by astrocytes at the DREZ has been proposed to result from either the formation of a physical barrier, or the activation of physiological stop mechanisms in the axons (Liuzzi, F.J., and Lasek, R.J., 1987, Science 237:642-645). If such a physiological stop mechanism is activated by astrocytes at the DREZ, why is it not activated by mature forebrain astrocytes in vitro? One possibility is that DREZ astrocytes but not forebrain astrocytes possess the capacity to inhibit axon outgrowth. While regional differences in CNS astrocytes have been observed (Chamak, B., et al., 1987, J. Neurosci. 7(10):3163-3170), it seems more likely that the

total inhibition of axon growth induced by the astrocytes of the DREZ may be the result of an architecturally different environment (Freed, W.M., et al., 1985, Science 227:1544-1552; Reier, P.J., 1986, in Astrocytes: Cell Biology and Pathology of Astrocytes, Fedoroff, S. and Vernadakis, A. (eds.), Academic Press, pp. 263-324). Thus, at the DREZ, growing axons are presented with a three dimensional matrix of astrocytes, while in culture growing axons are presented with only a two dimensional astrocyte substrate.

Although the molecular mechanism of inhibition of axon growth through a region of gliosis or at the dorsal root entry zone in the adult animals is unknown, one possibility is that it results from changes in the cell adhesion properties of the astrocytes. Considerable axon outgrowth may only occur when cell-cell adhesion was optimal. The finding that with maturation there appears to be a dramatic reduction in the adhesivity of astrocytes for neuronal cell bodies suggests that a decrease and not an increase in neuron/glial interactions is responsible for the reduction of axon growth through gliosis in the adult CNS.

A number of observations suggest that mature astrocytes do in fact represent a less adhesive substrate for growth cones than immature activated astrocytes. For instance, we have observed that neurons extending processes over non-confluent cultures of immature astrocytes invariably remained in association with the astrocyte surface rarely growing onto the poly-l-lysine coated coverslip, while neurons extending processes over non-confluent cultures of mature astrocytes frequently grew off the surface of the cells and onto the surrounding polylysine substrate. Such observations suggest that the surface of immature astrocytes represented a preferred substrate for axon growth

compared to the surrounding surface, while immature astrocytes did not. Furthermore, when brain explants were plated on the surface of immature cultured astrocytes, neurite outgrowth appeared to be predominantly nonfasciculated. In contrast, when brain explants were plated onto the surface of mature cultured astrocytes, neurite outgrowth occurred in a predominantly fasciculated fashion. Since the degree of axon fasciculation has been suggested to reflect the relative adhesivity of a substrate (Rutishauser, U., 1984, Nature 310:594-587), such that a more adhesive substrate results in defasciculated neurite outgrowth while a less adhesive substrate results in fasciculated neurite outgrowth (Rathjen, F.G., et al., 1987, J. Cell Biol. 104:343-353), these results also suggest that immature astrocytes represent a more adhesive substrate for the growing axon than do mature astrocytes.

In conclusion, we have shown that in vitro the ability of astrocytes to support CNS axonal elongation and bind CNS neurons is reduced as the astrocyte matures. We propose that this reduction in the capacity of mature astrocytes to support CNS neurite elongation, possibly through reduced adhesivity, may be an important component in limiting the extent of axonal regeneration in the adult mammalian CNS.

10. IN VITRO ANALYSES OF ASTROCYTE CELL INTERACTIONS AT DIFFERENT AGES

We have developed an in vitro model in which cells responding to trauma in the immature and mature CNS can be isolated, placed into serum-free culture and characterized. By implanting nitrocellulose filters into the brains of neonatal and adult rats under different conditions, we were able to harvest populations of cells responding to trauma in the neonate (critical period

implant), in the adult (scar implant), and implants that have remained in vivo past the critical period (post-critical period implant). Upon placement in culture, we have found that astrocytes represent the majority of cells occupying both the critical period and post-critical period implants, whereas fibroblasts and macrophages represent the majority of cells in the glial-fibroblastic scar. The morphologies of the astrocytes on the surface of the different implants, after three days in culture, differs markedly - the critical period astrocytes exhibiting a more ordered distribution compared to the haphazard arrangement of astrocyte processes on the surface of the post-critical and scar implants. After migration from the implant, critical period astrocytes assume an epithelioid morphology and cluster together setting up definite boundaries between themselves and the endothelial cells. In contrast, post-critical period astrocytes exhibit a more elongated morphology under the same culture conditions and appear to be randomly dispersed among the endothelial cells. The scar astrocytes exhibit a wide range of morphologies and although they tend to cluster, do not exhibit the ordered association seen with the critical period astrocytes. We propose that the plasticity of the neonatal astrocytes and the rapid and ordered cellular response seen in vitro reflect the ability of the immature CNS in vivo to respond to injury without the formation of a glial-fibroblastic scar.

11. INDUCED REGENERATION OF CUT DORSAL ROOT FIBERS INTO ADULT RAT SPINAL CORD

Glial scar formation and inhibition by white matter are thought to impede sensory axons from regenerating across the dorsal root entry zone (DREZ) into the adult rat spinal cord. In order to assess the

relative roles of these barriers, we unilaterally transected lumbar dorsal roots (L4-L6) and implanted the distal portion of L4 medially into the white matter of the dorsal columns or laterally just superficial to the gray matter of the dorsal horn. After three weeks, anterograde labelling with HRP demonstrated that many axons entered the spinal cord from laterally-implanted roots. Some of these axons entered the gray matter and formed terminal fields with synaptic boutons. Other axons extended into the white matter of the dorsal columns for variable distances. The placement of a specially designed Millipore filter, coated with embryonic astrocytes, just medial to the implanted root increased regeneration into the gray matter. Successful regeneration of axons was associated with a localized and limited inflammatory response near the sites of ingrowth. Axonal regeneration thus appears to be enhanced by the lateral placement of a transected sensory root, the use of an immature activated astrocyte-coated polymer, and the presence of a limited inflammatory response.

12. ANALYSIS OF NEONATAL AND ADULT RAT OLFACTORY BULB GLIAL CELL LINES

The rat olfactory bulb is reinnervated throughout life unlike other regions of the central nervous system, where glial differentiation effects loss of plasticity. In vitro, the differentiated phenotype of glial cells can be preserved by cell immortalization via oncogene transduction. We have used this approach to facilitate a study of the roles of glia in the innervation of the olfactory bulb.

Immortalized cell lines of ensheathing cells have been established from cultures of rat neonatal and adult olfactory bulbs and characterized concurrently with analogous primary cells. Two distinct phenotypes are

evident within both neonatal and adult primary cultures: stellate, type I astrocyte-like, and fusiform (Anat. Record 210:385; Dev. Biol. 130:237) cells, each containing glial filaments. Both phenotypes are also present in uncloned immortalized cultures of neonatal and adult bulb.

Neurite outgrowth over monolayers of mature (adult) and neonatal cerebral cortical astrocyte cultures were compared to outgrowth over monolayers of adult olfactory fusiform and stellate $GFAP^+$ clones, using a 24-hour neurite outgrowth assay with stage 31 chick retinal neurons. Mean neurite length was equivalent for adult fusiform olfactory cell lines (immortalized) and both the primary and immortalized immature cortical cells. In contrast, the mean length for the mature cortical astrocyte cells (both immortalized and primary cells) was reduced to the same extent as the reduction seen in immature cortical astrocyte cultures relative to primary immature cortical astrocyte cultures. These results suggest that: 1) the neurite promoting properties of glial cells are preserved through immortalization, and 2) the neurite promoting properties of adult olfactory glial cells and neonatal cerebral astrocytes are similar.

13. EXAMPLE: IMMATURE TYPE-1 ASTROCYTES SUPPRESS GLIAL SCAR FORMATION, ARE MOTILE AND INTERACT WITH BLOOD VESSELS

13.1. MATERIALS AND METHODS

13.1.1. PREPARATION OF PURIFIED TYPE-1 RAT CORTICAL ASTROCYTES

Purified type-1 rat cortical astrocytes were prepared as described in Smith et al. (1990, Dev. Biol. 138:377-390). Briefly, cerebral cortices of newborn mice were isolated, meningeal tissue removed and the cells dissociated using 0.025% trypsin for 30 minutes. Cells were pelleted at 1000g, resuspended in 5 ml of 50% astrocyte conditioned-medium in DMEM containing 10% FCS,

and plated into a polylysine coated $75cm^2$ flask at a density of $2x10^6$ cells/flask. Following 4-6 hours incubation at 37°C the majority of neurons, oligodendrocytes, type-2 astrocytes and O-2A progenitor cells were removed from the flask with the medium following shaking. To remove any remaining contaminating cells, the shaking procedure was repeated every day for 2-3 days. Purified populations of immature type-1 astrocytes were obtained from the residual cultures no later than 3-4 days in culture.

To obtain pure populations of mature type-1 astrocytes, cells were grown to confluence, replated and two days later treated with a 2-day pulse of cytosine arabinoside ($2.5x10^{-5}$ M) to remove any fibroblasts. Mature astrocytes were harvested from such cultures following an additional 28-day culture period.

The purity of both immature and mature type-1 astrocyte cultures was assayed by indirect immunofluorescence using a variety of cell-type-specific markers including antibodies against GFAP to identify astrocytes (Pruss, 1979, Nature 280:688-690) fibronectin, to identify fibroblasts, monoclonal antibody A2B5 (Eisenbarth et al., 1979, Proc. Nat. Acad. Sci. U.S.A. 76:4913-4917) to identify O-2A progenitor cells and type-2 astrocytes, and galactocerebroside, to identify oligodendrocytes (Ranscht et al., 1982, Proc. Nat. Acad. Sci. U.S.A. 70:2709-2713), as described in Smith et al., 1990, Dev. Biol. 138:377-390. In both cultures more than 95% of the cells had the characteristics of type-1 astrocytes (See Smith et al., 1990, Dev. Biol. 138:377-390).

13.1.2. LABELING OF CULTURED ASTROCYTES AND IMPLANTATION PROCEDURE

To allow identification of transplanted astrocytes in the host brain, cells were labeled in culture before being placed on the nitrocellulose implant. For light microscopy, 20μl of 0.07 μm FITC coated microspheres (Polysciences Inc.) were mixed with 10mls of DMEM-F12 and added to a flask containing astrocyte cultures for 6 hours at 37°C. Excess microspheres were removed by washing in DMEM-F12 and the cultures incubated overnight in serum-free N2 medium (Bottenstein et al., 1979, Proc. Nat. Acad. Sci. U.S.A. 79:514-517). For electron microscopy, concentrated 17nm colloidal gold conjugated to BSA was mixed with DMEM-F12 to yield a light red color. 10ml of this suspension was added to the culture well and cells incubated overnight at 37°C to facilitate internalization. Excess colloidal gold was removed by extensive washing in DME and the cells were incubated in serum-free media for 24 hours at 37°C prior to seeding onto nitrocellulose implants.

To coat nitrocellulose implants, astrocytes were removed from culture flasks and washed through three cycles of centrifugation and resuspension in 1ml DMEM. In the final wash cells were resuspended in only 200μl DMEM. Cell number and viability was determined using trypan blue, and 20μl of astrocyte suspension was seeded on to an individual implant at a density of 10^6 cells/ml (approximately $2x10^4$ astrocytes/implant). The cell suspension which generally formed a droplet on the implant surface was incubated for 2 hours at 37°C, and the implant was then flooded with 100μl of serum-free (N2) medium and incubated overnight. The following day, astrocyte-coated implants were transplanted into adult (P 31) rats using a similar method to that described in Silver et al. (1983, Science 220:1067-1069) and Smith et

al. (1986, J. Comp. Neurol. 251:23-43). Briefly, host animals were anesthetized with 1.2ml/kg body weight of a mixture of 52.6% Ketamine (Aveco) and 8.8% Xylazine (Mobay) in sterile saline, and a small incision made through the skin horizontally between the eyes. Skin was retracted and the surface of the skull cleaned of attached tissue. A small hole was then drilled in the skull and the implant with attached astrocytes inserted directly into the forebrain. The wound was closed and the animals allowed to recover for at least 9 days before sacrifice. Control animals received implants treated as above with the exception that either no astrocytes were added to the surface, or the coating cells were killed by heating to 60°C for 15 minutes prior to implantation.

13.1.3. TISSUE PREPARATION

For immunofluorescence analysis, 6 rats that had received successful implants coated with either immature or mature astrocytes were assayed. Nine days after implantation, four animals in each group were anesthetized by nembutal injection and sacrificed by cardiac perfusion, while two animals in each group were sacrificed by the same procedure 2 months after implantation. For light microscopy animals were perfused with 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4, the region of brain containing the implant removed, post-fixed overnight at 4°C, infused with 1M sucrose, frozen,and 10μm cryostat sections collected on gelatin coated slides. Antibody staining was performed at room temperature for a minimum of 4 hours with primary antibodies at the following concentrations. Rabbit anti-GFAP (1:1000), Rabbit-anti-Laminin (1:100) and secondary antibody Goat-anti-rabbit conjugated to fluorescein (Cappel) (1:200). All antibody incubations were performed in dilution buffer comprised of 1% triton

X100, 5% BSA, 10% normal goat serum (NGS) in 0.1M phosphate at pH 7.4. Specificity of labelling was confirmed by deletion of the primary antibody or substitution of the primary antibody with normal rabbit serum. Slides were examined on a Nikon Optiphot microscope equipped with epifluorescence and photographed on TriX film at 400ASA.

For electron microscopy, anesthetized animals were perfused with a mixture of 3% glutaraldehyde and 5% paraformaldehyde in 0.1M phosphate buffer, the region of brain containing the implant removed, post fixed in 2% Osmium tetroxide for 4 hours, and "en bloc" stained in saturated aqueous uranyl acetate overnight at 4°C. Samples were then dehydrated through graded alcohols and embedded in Spurr resin. Gold colored sections were cut on an LKB microtome and examined on a Jeol 100CM microscope at 80KV without further staining.

13.1.4. ANALYSIS OF BLOOD VESSEL PERMEABILITY FOLLOWING ASTROCYTE TRANSPLANTATION

To determine the permeability of blood vessels in the region of the lesion, animals that had received implants coated with either immature or mature astrocytes were allowed to survive for a further 9 days. A 2% solution of Evans Blue dye (21ml/Kg body weight) (Eastman Kodak Inc.) in sterile saline was then introduced into the femoral vein following ether anesthesia. The wound was sealed and the animals allowed to survive for either 15 minutes (1 animal each with immature and mature astrocyte coated implants) or 4 hours (3 animals in each class) post injection. Successful injections were monitored through the blue appearance of the eye and skin of the animal. Animals were then re-anesthetized and decapitated. Brains were removed and fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer for at

least 8 hours at 4°C. Brain regions c ntaining the implant were dissected from the rest of the CNS, infiltrated with 1M sucrose and 40μm thick frozen sections prepared as described supra. Dye diffusion was assayed by examining the sections using a rhodamine filter (Chan et al., 1983, Ann. Neurol. 13:625-631) and images recorded on Tri X film.

13.1.5. CELL MOTILITY ASSAY

To compare the capacity of immature and mature astrocytes to translocate in a homogenous environment, cells were plated on 12mm poly-L-lysine coated coverslips at a density of $1x10^3$ cells/coverslip. Cells were allowed to attach overnight, the coverslip transferred to the stage of a time lapse video microscope and their motility assayed. Cell motility was recorded using a Zeiss inverted microscope coupled to a video camera and time lapse recorder (Panasonic AG6060). For both populations of astrocytes, the percentage of cells showing any translocation, and the rate of individual cell locomotion was followed over a continuous 10 hour period at 1 hour intervals. Measurements of translocational rate were made from three different preparations, and the data from the analysis of a total of 100 cells in each population combined.

13.2. RESULTS

13.2.1. IMMATURE BUT NOT MATURE TYPE-1 RAT CORTICAL ASTROCYTES SUPPRESS GLIAL SCAR FORMATION

To compare glial scar formation in host rats that had received nitrocellulose implants coated with either immature or in vitro matured type-1 astrocytes, frozen sections through the implant and surrounding host tissue were labelled with antibodies against GFAP to

identify astrocytes, and laminin to localize basal lamina. In 4 of the 6 animals that received implants coated with immature astrocytes, the implant became closely incorporated into the parenchyma of the host CNS (FIGURES 25a and 25b). In the region of the implant there was only a slight increase in anti-GFAP compared with the rest of the CNS (FIGURE 25a) demonstrating a limited response by the host astrocytes. Similarly, using antibodies against laminin, clearly delineated blood vessels and some slight staining in the interior of the implant was observed (FIGURE 25b), but there was little staining in the host cortex around the implant. In the remaining 2 animals which received immature astrocyte coated implants, restricted areas around the implant showed both increased GFAP and basal-lamina associated laminin staining. However, such labelling was never generalized over the surface of the implant and may have resulted from a lack of viable transplanted astrocytes in such regions.

By contrast, all animals that received implants coated with mature type-1 astrocytes displayed dramatic increases in both GFAP (FIGURE 25c) and laminin (FIGURE 25d) expression in the region of the implant. The laminin appeared to be associated with regions of extensive basal lamina deposition, and a dense glial scar. The extent of glial scar formation seen following transplantation of mature astrocyte coated implants was similar to that seen following implantation of either non-coated nitrocellulose implants or those coated with heat-killed astrocytes, all of which showed extensive glial scar formation consistent with previous studies (Smith et al., 1986, J. Comp. Neurol. 251:23-43). In addition to the formation of an extensive glial scar, a large number of macrophages were found in the region of the implants coated with mature but not immature type-1

astrocytes. These observations suggest that immature but not mature type-1 rat cortical astrocytes have the capacity to suppress glial scar formation when transplanted into an adult rat host CNS.

13.2.2. IMMATURE BUT NOT MATURE TYPE-1 ASTROCYTES ASSOCIATE WITH BLOOD VESSELS IN THE HOST CORTEX

To examine the fate of transplanted astrocytes in the adult host cortex, cells were labelled with either FITC coated latex microspheres, or colloidal gold particles prior to transplantation. Both immature and mature astrocytes ingested the labels although generally mature astrocytes were more heavily labelled than immature astrocytes after a similar time interval. To ensure that labelling did not reduce cell viability and that the label was not transferred between cells the following experiments were performed: Separate fractions of either immature or mature astrocytes were labelled with FITC or RITC coated microspheres, and equal amounts of the two fractions mixed and grown under standard conditions. After 2, 4 and 7 days cultures were fixed and examined under fluorescein and rhodamine optics. More than 98% of the cells contained one or the other label and in all cases individual astrocytes exclusively contained either FITC or RITC microspheres. To test colloidal gold labelling, a small number of labelled astrocytes were plated in the center of a coverslip and allowed to attach for 4 hours. An excess of unlabelled astrocytes from the same cultures were then plated over the remaining coverslip surface, and the location and approximate number of labelled cells determined 3, 5, 7 and 9 days later. In all cases, the approximate number of labelled astrocytes was similar at each time point and labelled cells were generally located in the central

portion of the coverslip in the general area where they had been originally plated. These observations indicate that both labelling paradigms had little effect on cell viability and that widespread transfer of label between cells did not occur.

In sections of host brains that had received implants coated with labelled immature astrocytes, fluorescently labelled cells were found both on the surface of the implant and in discrete locations throughout the host CNS. Immunostaining of such sections with anti-GFAP antibodies suggested that the labelled cells were GFAP+ astrocytes (FIGURES 26a and 26b). Clusters of labelled cells in the surrounding host CNS were found predominantly around blood vessels as identified by laminin immunoreactivity (FIGURES 26c and 26d). At higher magnification, it could be seen that those astrocytes which remained associated with the surface of the implant had processes penetrating into the interior of the implant, a characteristic of immature astrocytes (FIGURES 26e, 26f, and 26g) while cells which had migrated from the implant were closely associated with blood vessels in the host CNS.

However, the resolution provided by this type of analysis could not distinguish between the migration of labelled astrocytes, or the phagocytosis of debris from GFAP+ labelled cells by macrophages, and subsequent migration of these macrophages through the host CNS. To examine in more detail the characteristics of the labelled cells, thin sections from colloidal- gold-labelled immature astrocyte preparations were examined in the electron microscope. The majority of labelled cells both close to the surface of the implant, and within the host CNS had the morphological characteristics of astrocytes. In the host parenchyma, labelled astrocytes were generally seen in close association with well formed

blood vessels (FIGURES 27a, 27b and 27c) and in a few cases a single astrocyte had close association with more than one blood vessel (FIGURES 27d and 27e). Not all the blood vessels in the region of the implant were associated with labelled astrocyte processes. However, since immature astrocytes were only lightly labelled, and the label was particulate in the cytoplasm, more blood vessels may have been associated with labelled astrocyte processes than were detectable in any single section.

In sections of host brains which received mature astrocyte coated implants, both light microscopy and electron microscopy failed to reveal any labelled mature astrocytes within the host brain parenchyma. All the labelled cells were found on the surface of the implant (FIGURE 28a), or in the surrounding glial scar. Furthermore, many of the cells which contained label had morphological characteristics of macrophages and not astrocytes (FIGURES 28b and 28c). These label containing macrophages also contained large amounts of astrocyte cell debris (FIGURES 28b and 28c), suggesting that survival of mature astrocytes was significantly less than that of immature astrocytes following transplantation. Although numerous blood vessels were present in the region of the mature astrocyte coated implant, such vessels were not completely surrounded by astrocyte processes as they were in the region of implants coated with immature astrocytes. These observations suggest that immature type-1 astrocytes survive better in the host animal than mature astrocytes and these immature cells have the capacity to migrate from the surface of the implant and become associated with blood vessels in the surrounding tissue.

13.2.3. BLOOD VESSEL PERMEABILITY IN THE IMPLANT REGION

CNS blood vessels are known to have different permeability characteristics when compared with blood vessels in other tissues (Reese et al., 1967, J. Cell Biol. 34:207-217; Stewart et al., 1981, Dev. Biol. 84:183-192). The close association of astrocyte endfeet with blood vessels suggests that astrocytes may influence vascular permeability, and recent studies provide evidence for participation of type-1 astrocytes in this function (Janzer et al., 1987, Nature 325:253-257). Since immature astrocytes became associated with blood vessels in the region of the implant, while mature astrocytes did not, the permeability characteristics of the blood vessels around the two types of implant was examined.

In those animals which received implants coated with immature astrocytes, blood vessels in the region of the implant were clearly discernible from the surrounding tissue. Little diffusion of the dye from these vessels was seen either 15 minutes or 4 hours following injection (FIGURES 29a and 29b). By contrast, in those animals that received implants coated with mature astrocytes, considerable dye diffusion was seen 4 hours after injection (FIGURES 29c and 29d), while little diffusion was seen after a 15 minute post injection interval. In both cases, the implant and surrounding region contained some autofluorescent cells, and blood vessels away from the region of implant were clearly impermeable to the tracer. These observations indicate that the association of the immature astrocytes with blood vessels around the implant contribute to the reformation of the blood brain barrier in that region.

13.2.4. IMMATURE TYPE-1 ASTROCYTES ARE MORE MOTILE THAN MATURE TYPE-1 ASTROCYTES

Because immature but not mature astrocytes frequently migrated from the surface of the implant and became associated with blood vessels in the host brain, it seemed likely that immature astrocytes were the more motile cells. To determine if this apparent difference in motility was an intrinsic property of immature astrocytes, or resulted from their less restricted passage through the CNS, the ability of immature and mature type-1 astrocytes to translocate over a poly-L-lysine substrate was compared in vitro.

In both astrocyte populations the majority of cells showed extensive cellular activity including continuous extension and retraction of filopodia and formation of surface ruffles. However, when the rate of cell translocation was compared between the two populations of cells, immature astrocytes were found to translocate more than twice as rapidly as mature astrocytes (Table VII). Both immature and mature astrocytes translocated in an irregular manner. Short periods of translocation were interspersed with stationary periods in which the cell continued to display filopodial and ruffling behavior. One of the major differences between immature and mature astrocytes was that individual immature cells spent longer periods of time actively translocating, than localized ruffling compared to mature cells. This difference accounted for the greater overall motility rate observed with immature astrocytes, and the results indicate that on a homogenous two-dimensional substrate, immature astrocytes are intrinsically more motile than mature astrocytes.

13.3. DISCUSSION

A purified population of type-1 astrocytes from the neonatal rat forebrain has the capacity to suppress glial scar formation when transplanted into an adult host in their immature form, but lose this capacity as they mature in vitro. Thus, it seems likely that the functional changes reported following transplantation of enriched populations of variously aged astrocytes in other systems (Smith et al., 1986, J. Comp. Neurol. 251:23-43; Smith et al., 1988, "Progress in Brain Research", 78 Elsevier pp. 353-361, Gash DM, Sladek JR. eds.; Schreyer et al., 1987, Dev. Brain Res. 35:291-299) are due in large part to a maturational change in type-1-like astrocytes.

Using labelled cells, immature but not mature type-1 astrocytes were shown to migrate away from the surface of the implant and become associated with blood vessels in the host CNS. The difference in migratory capacity between immature and mature astrocytes may result from a variety of factors. Immature astrocytes appear to be intrinsically more motile than mature astrocytes (Duffy et al., 1982, Exp. Cell. Res. 139:145-157, Table VII). Further, short term adhesion assays have shown that immature astrocytes are more adhesive to neurons than are mature astrocytes and this increased neuronal interaction may allow immature astrocytes to translocate more effectively through the host neuropil. In similar assays, mature astrocytes have an adhesive preference for each other and form more intercellular junctions than do immature astrocytes (Sipe, J. C., 1976, Cell Tissue Res. 170:485-490. This increased cellular interaction between adjacent mature astrocytes may inhibit their ability to release themselves from neighboring astrocytes on the implant surface and subsequently migrate through the host neuropil. In

addition, the dense basal lamina in the glial scar, formed in part, by mature astrocytes may further inhibit their migration (Bernstein et al., 1985, Brain Res. 327:135-141).

The ability of immature astrocytes to migrate through the CNS has been shown both following transplantation of fetal tissue or purified neonatal cells (Lindsay et al., 1984, Neurosci. 12:513-530; La Chapelle et al., 1984, Dev. Neurosci. 6:325-334; Smith et al., 1988, "Progress in Brain Research", 78 Elsevier pp. 353-361, Gash DM, Sladek JR. eds.), and after lesions to the neonatal CNS (Ali-Ali, et al., 1988, Glia 1:211-219). Frequently these migrating astrocytes appear associated with blood vessels in the host CNS (Lindsay et al., 1984, Neurosci. 12:513-530; Ali-Ali et al., 1988, Glia 1:211-219; Lawrence et al., 1984, Neurosci. 12:745-760). The finding here that immature but not mature transplanted type-1 astrocytes become associated with host blood vessels, may influence the subsequent permeability characteristics of the blood vessels that invade the region of the implant following injury.

Type-1 astrocytes have been proposed to contribute to the formation of the blood brain barrier (Janzer et al., 1987, Nature 325:253-257). In studies using purified neonatal type-1 astrocytes placed on the chorioallantoic membrane of chick embryos, the blood vessels in the region of the astrocyte aggregate were shown to have the permeability characteristics of CNS blood vessels and not of the surrounding tissue (Janzer et al., 1987, Nature 325:253-257). In the present study, following transplantation of immature astrocytes, blood vessels in the region of the implant were found to substantially and surprisingly retard the diffusion of Evans blue compared to the extensive diffusion of dye observed in implants which had received mature

astrocytes, suggesting that the blood brain barrier had b en reformed by the immature astrocytes. Under normal conditions, the blood brain barrier participates in the isolation of the CNS, and when compromised, a continued immune response directed against cellular elements of the CNS may reduce the survival of both transplanted and host tissue (Berry et al., 1974, Brit. Med. Bull. 30:135-140). Therefore, the rapid reformation of the blood brain barrier by the transplanted young astrocytes may contribute to their suppression of glial scar formation in the adult animal by restricting the immune response.

The survival of transplanted astrocytes differed substantially between the immature and mature cells. In all cases, transplantation of mature astrocytes resulted in greater cell death than transplantation of immature astrocytes. Consequently at the ultrastructural level, labelled astrocyte cell debris was more frequently seen in macrophages following mature rather than immature astrocyte transplantation. The reason for this difference is unclear. It seems unlikely that the labelling procedures themselves reduced mature astrocyte viability since labeled astrocytes survived for long periods of time both in vitro and in other studies following transplantation into the immature CNS.

Mature astrocytes may become a target for the haematogenous macrophages which, in the absence of an intact blood brain barrier have comparatively free access to the neuropil surrounding the implant. Changes in the expression of cell adhesion (Smith et al., 1990, Dev. Biol. 138:377-390), the major histocompatability antigens (Birbaum et al., 1986, J. Neuroimmunol. 12:225-233), are known to occur in astrocytes during their maturation and may contribute to their increased immunogenicity. Thus, while immunological tolerance to immature astrocytes is induced prior to the formation of the blood brain

barrier, the subsequent maturational changes in astrocytes may later render them immunogenic when exposed to cells of the immune system following CNS injury.

The results of this study are consistent with the hypothesis that immature type-1 astrocytes reduce adult glial scar formation by rapidly migrating from the lesion site, associating with host blood vessels and subsequently inhibiting the diffusion of macromolecules, and possibly, infiltration of non-neural cells into the lesion site. By contrast, mature astrocytes fail to migrate and associate effectively with blood vessels; instead they contribute locally to glial scar formation, and may themselves be a target for invading non-neural cells.

TABLE VII

CELL POPULATION	RATE OF MOTILITY
Immature astrocytes	8.9+/-2 μm/hr.
Mature astrocytes	3.6+/-0.4 μm/hr.

Cells were prepared as described in the methods and their motility assayed over a 10 hour period. Measurements were made at 1 hour intervals. For both cell groups the motility rate is the mean+/- standard deviation, taken from 100 cells in each class obtained from three different preparations.

14. DEPOSIT OF MICROORGANISM

Murine embryonic astrocyte cell line mEASTR-2 (mouse embryonic astrocyte clone number two) was deposited with the American Type Culture Collection, Rockville, Maryland, on February 1, 1989, and was assigned accession number CRL 10020.

The present invention is not to be limited in scope by the cell line deposited since the deposited embodiment is intended as a single illustration of one aspect of the invention and any cell lines which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method for promoting central nervous system axon regeneration comprising:

(a) providing olfactory bulb glial cells; and

(b) administering an effective amount of the olfactory bulb glial cells to damaged axons to promote axon regeneration.

2. The method according to claim 1 which further comprises before administering the olfactory bulb glial cells, substantially purifying the olfactory bulb glial cells.

3. The method according to claim 1, in which the olfactory bulb glial cells are immortalized.

4. The method according to claim 1, in which the damaged axons are forebrain axons.

5. The method according to claim 1, in which the damaged axons are commissural axons.

6. The method according to claim 1, in which the damaged axons are spinal cord axons.

7. The method according to claim 6, in which the spinal axons are sensory axons.

8. The method according to claim 1, in which the damaged axons are in the dorsal root entry zone of the spinal cord.

9. A method for reducing glial scar formation in damaged central nervous system tissue comprising:

(a) providing olfactory bulb glial cells; and

(b) administering an effective amount of the olfactory bulb glial cells to damaged tissue of the central nervous system to reduce glial scar formation.

10. The method according to claim 9 which further comprises before administering the olfactory bulb glial cells, substantially purifying the olfactory bulb glial cells.

11. The method according to claim 10, in which the olfactory bulb glial cells are immortalized.

12. The method according to claim 9, in which the damaged tissue of the central nervous system is in the brain.

13. The method according to claim 9, in which the damaged tissue of the central nervous system is in the spinal cord.

14. The method according to claim 9, in which the damaged tissue is secondary to surgery.

15. The method according to claim 9, in which the damaged tissue is secondary to trauma.

16. The method according to claim 13, in which the damaged tissue of the central nervous system is in the dorsal root entry zone of the spinal cord.

17. A method for promoting blood vessel regeneration in the central nervous system comprising:

(a) providing olfactory bulb glial cells; and

(b) administering an effective amount of the olfactory bulb glial cells to damaged blood vessels in the central nervous system to promote blood vessel regeneration.

18. The method according to claim 17, in which the olfactory bulb glial cells are immortalized.

19. The method according to claim 17 which further comprises before administering the olfactory bulb glial cells, substantially purifying the olfactory bulb glial cells.

20. The method according to claim 17, in which the damaged blood vessels are in the brain.

21. The method according to claim 17, in which the damaged blood vessels are in the spinal cord.

22. The method according to claim 21, in which the damaged blood vessels are in the dorsal root entry zone of the spinal cord.

23. A method for treating a patient with nerve damage comprising administering an effective amount of olfactory bulb glial cells to the patient.

24. The method according to claim 23, which further comprises before administering the olfactory bulb glial cells, substantially purifying the olfactory bulb glial cells.

25. The method according to claim 23 or 24 in which the olfactory bulb glial cells are administered by seeding the astrocytes onto or into an implant, and inserting the seeded implant into the patient.

26. The method according to claim 23 or 24 in which the olfactory bulb glial cells are administered within a gelatinous material.

27. A pharmaceutical composition comprising olfactory bulb glial cells; and a pharmaceutically acceptable carrier.

28. The pharmaceutical composition of claim 27, in which the olfactory bulb glial cells are substantially purified.

29. A method for reforming or reinforcing the blood brain barrier in central nervous system tissue which has been damaged in that the integrity of at least a portion of the blood brain barrier has been disrupted comprising:

(a) providing activated astrocytes; and

(b) administering an effective amount of the activated astrocytes to damaged tissue of the central nervous system to reform or reinforce the blood brain barrier.

30. The method according to claim 29, in which the activated immature astrocytes are stellate-shaped, GFAP-positive astrocytes.

31. The method according to claim 29, in which the activated immature astrocytes are embryonic through postnatal day 8 astrocytes.

32. The method according to claim 29 in which the activated astrocytes are olfactory bulb glial cells.

33. The method according to claim 29 which further comprises before administering the activated astrocytes, substantially purifying the activated astrocytes.

34. The method according to claim 29, in which the activated immature astrocytes are immortalized astrocytes.

35. The method according to claim 29 in which the central nervous system is damaged by surgery.

36. The method according to claim 29 in which the central nervous system is damaged by trauma.

37. The method according to claim 29 in which the central nervous system is damaged by malignancy.

38. The method according to claim 29 in which the central nervous system is damaged by infarction.

39. The method according to claim 29 in which the central nervous system is damaged by infection.

40. The method according to claim 29 in which the central nervous system is damaged by toxic agents.

41. The method according to claim 29 in which edema of central nervous system tissue is prevented.

42. The method according to claim 35 in which edema of central nervous system tissue is prevented.

43. The method according to claim 36 in which edema of central nervous system tissue is prevented.

44. The method according to claim 37 in which edema of central nervous system tissue is prevented.

FIG.1a

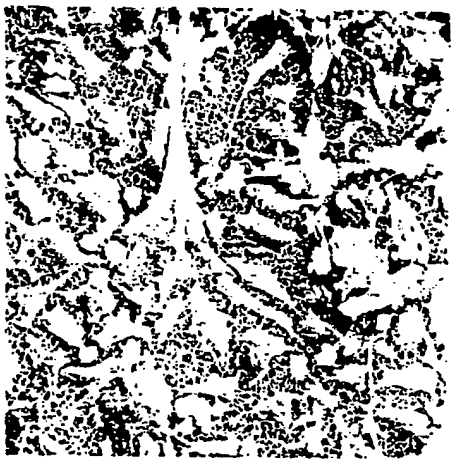


FIG.1b

FIG.1c

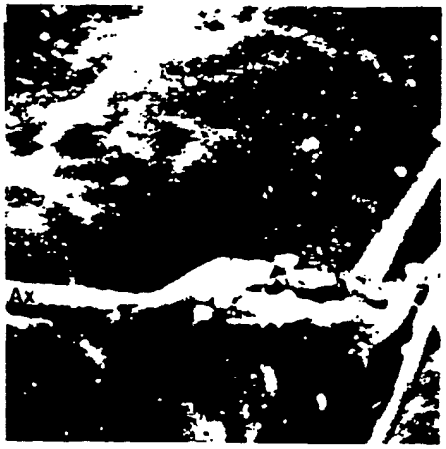


FIG.1d

FIG.2a

FIG.2b

FIG.2c

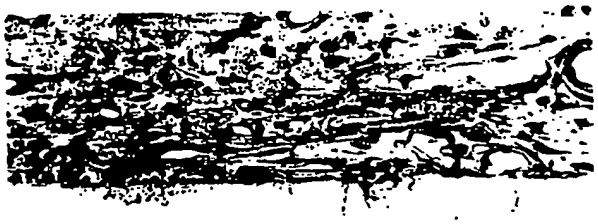


FIG.2d

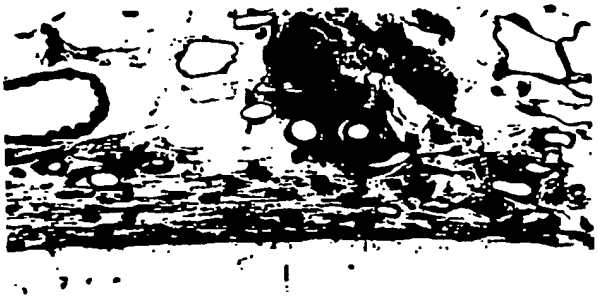


FIG.2e

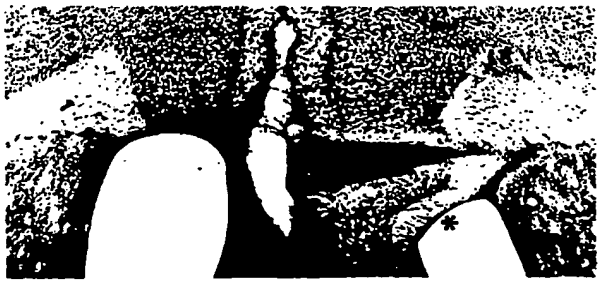


FIG.3a

FIG.3b

FIG.3c

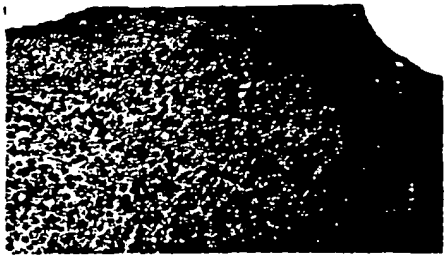


FIG.3d

FIG.3e

FIG.4a

FIG.4b

FIG.4c

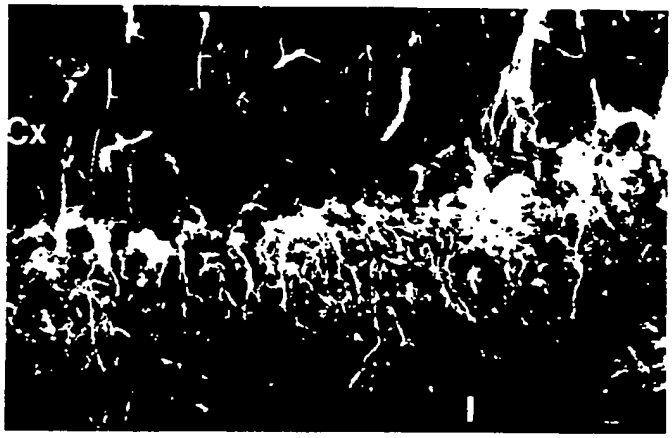


FIG.5a

FIG.5b

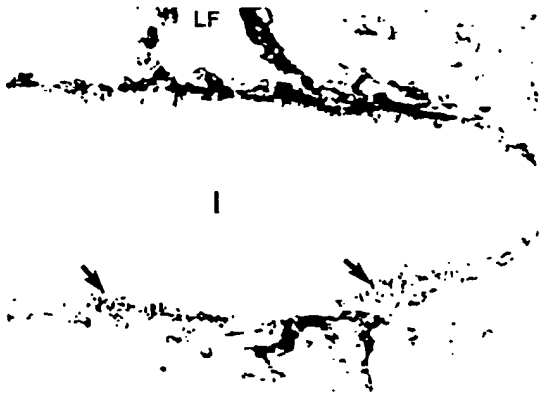


FIG.6a

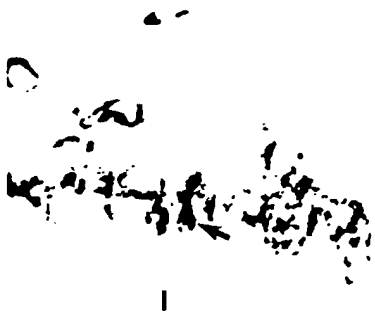


FIG.6b

FIG.6c

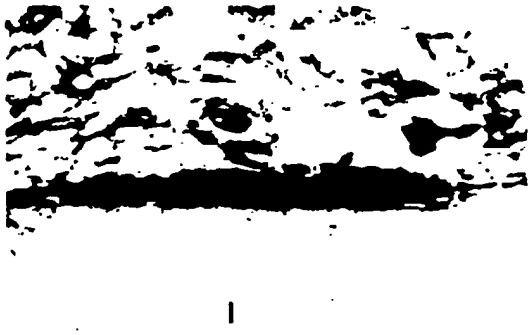


FIG.6d

FIG.6e

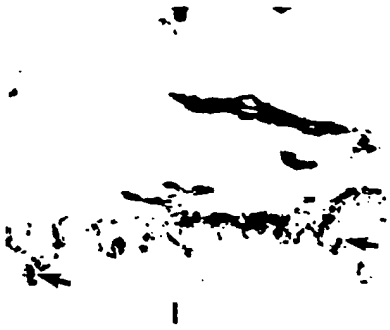


FIG.6f

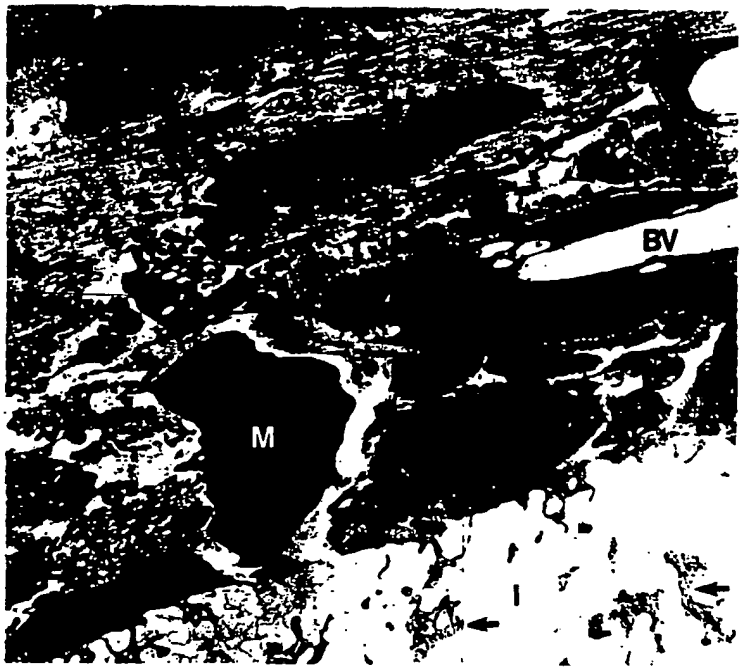


FIG.7a

FIG.7b

FIG.7c

FIG.7d

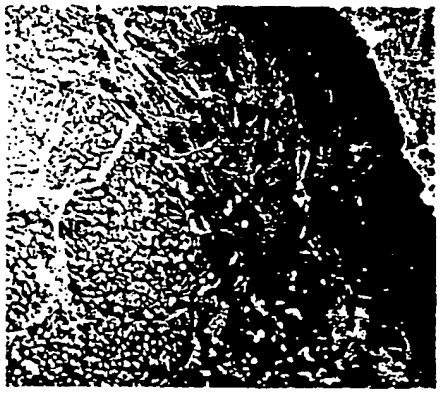


FIG. 8a

FIG. 8b

FIG. 8c

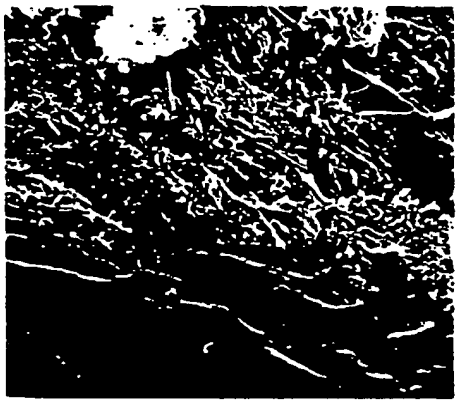


FIG. 8d

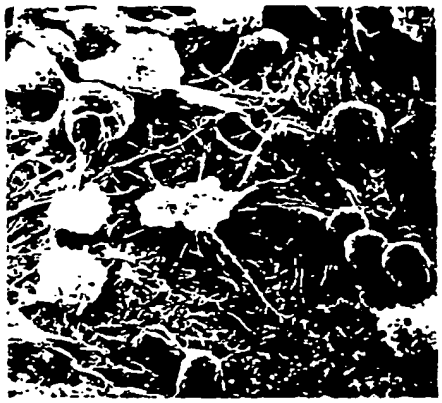


FIG. 8e

FIG. 8f

FIG.9a

FIG.9b

FIG. 10a

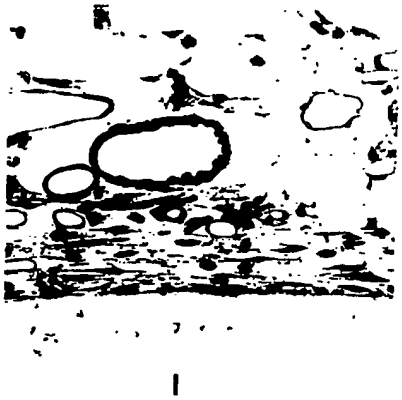


FIG. 10b

FIG. 10c

FIG. 10d

FIG.II

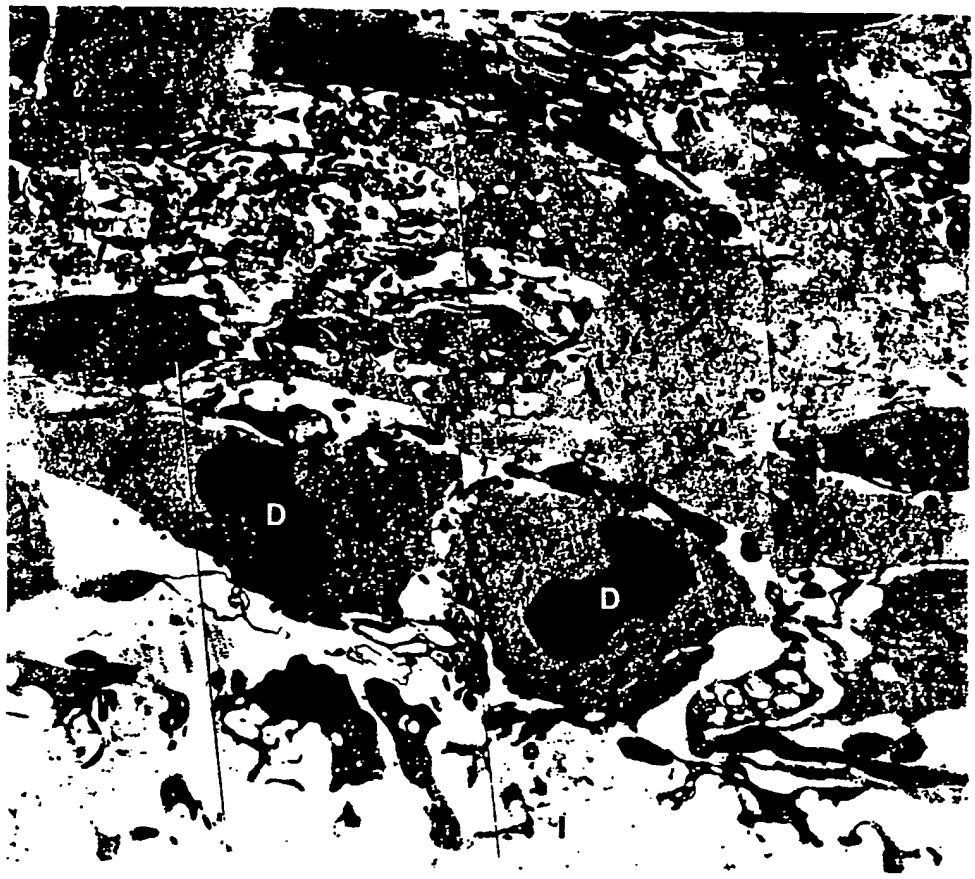


FIG. 12a

FIG. 12b

FIG. 12d

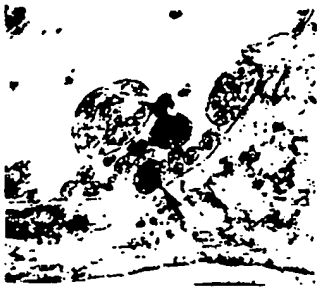


FIG. 12c

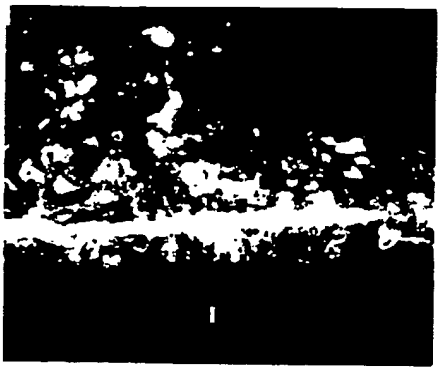


FIG. 13a

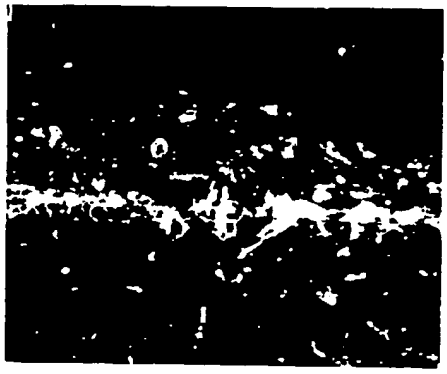


FIG. 13b

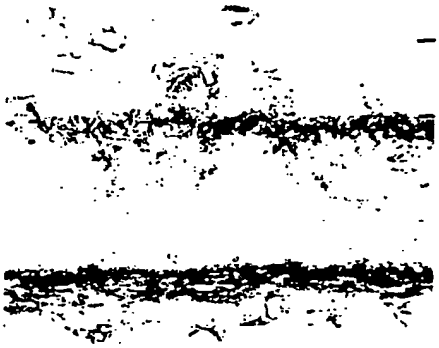


FIG. 13c

FIG. 13d

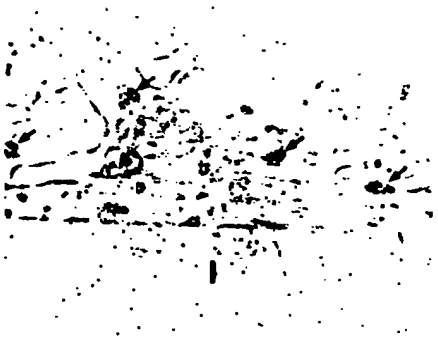


FIG. 13e

FIG. 13f

FIG. 14a

FIG. 14b

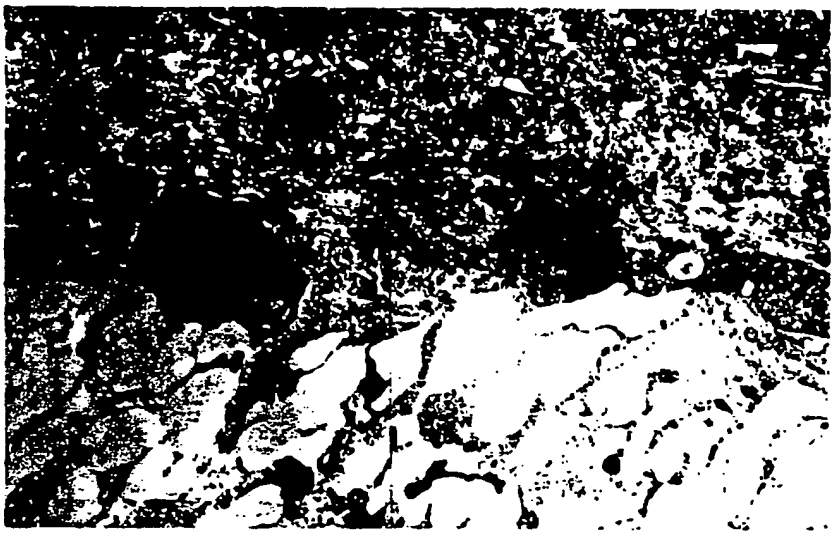


FIG. 15a

FIG. 15b

FIG. 16

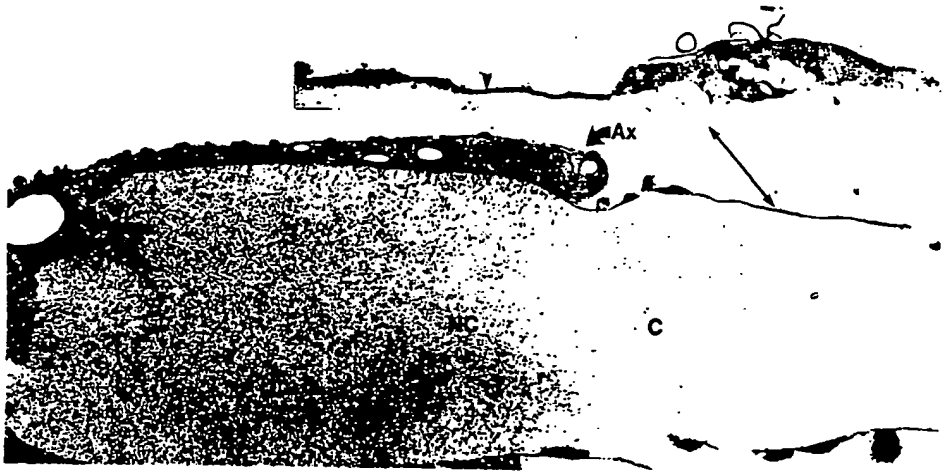


FIG. 17a

FIG. 17b

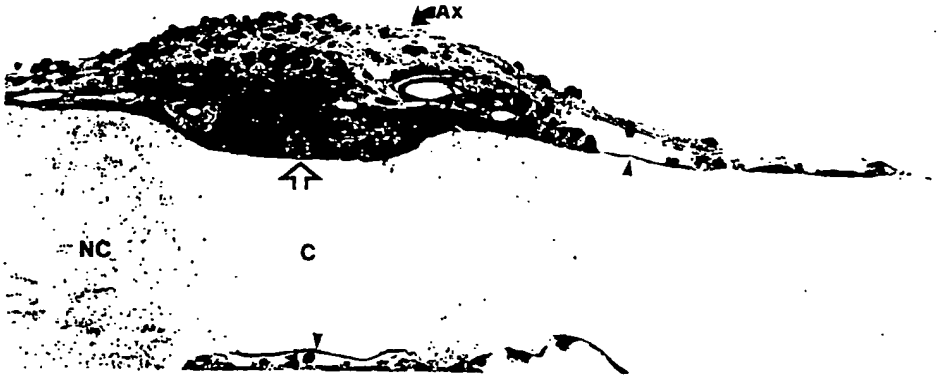
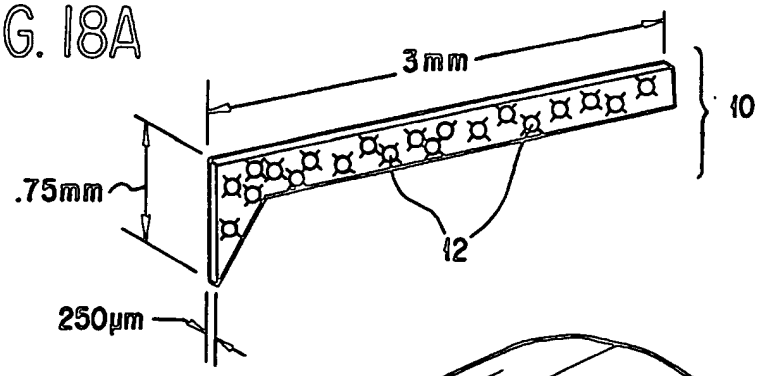


FIG. 17c

FIG. 18A



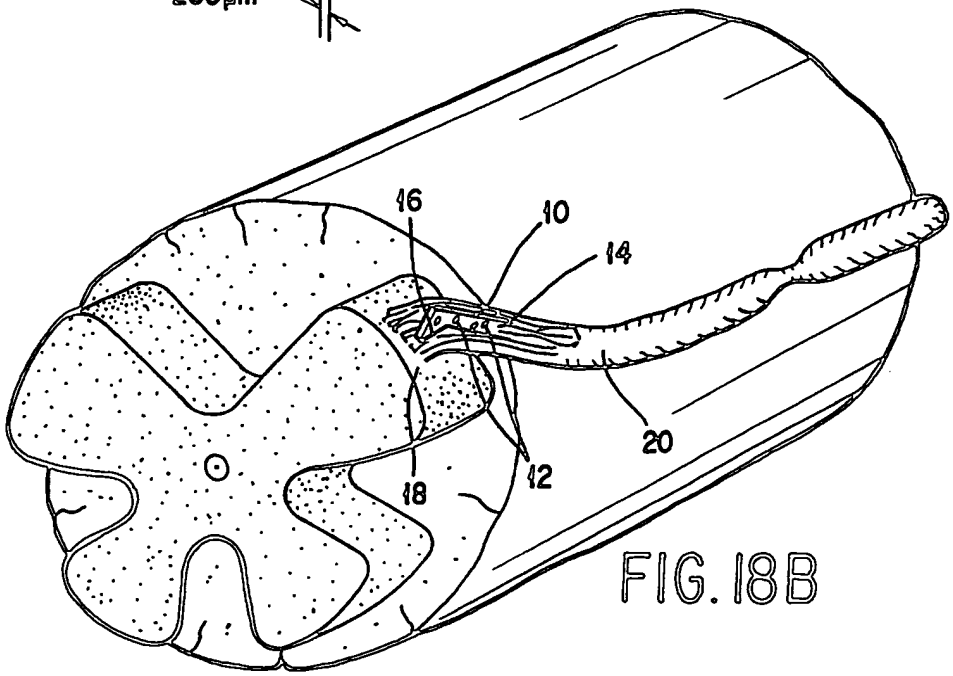


FIG. 18B

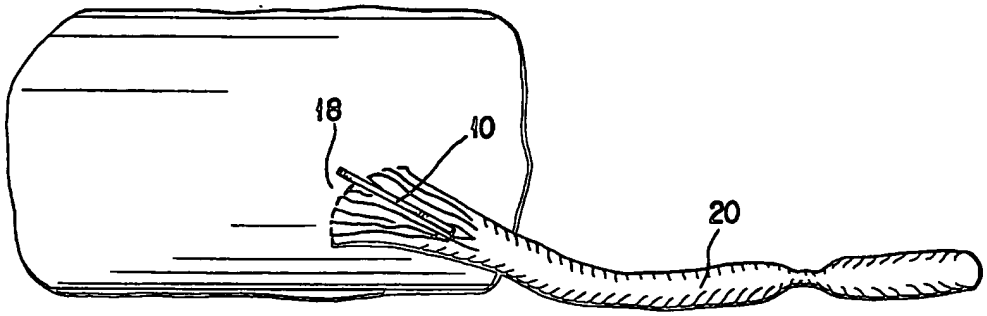


FIG. 18C

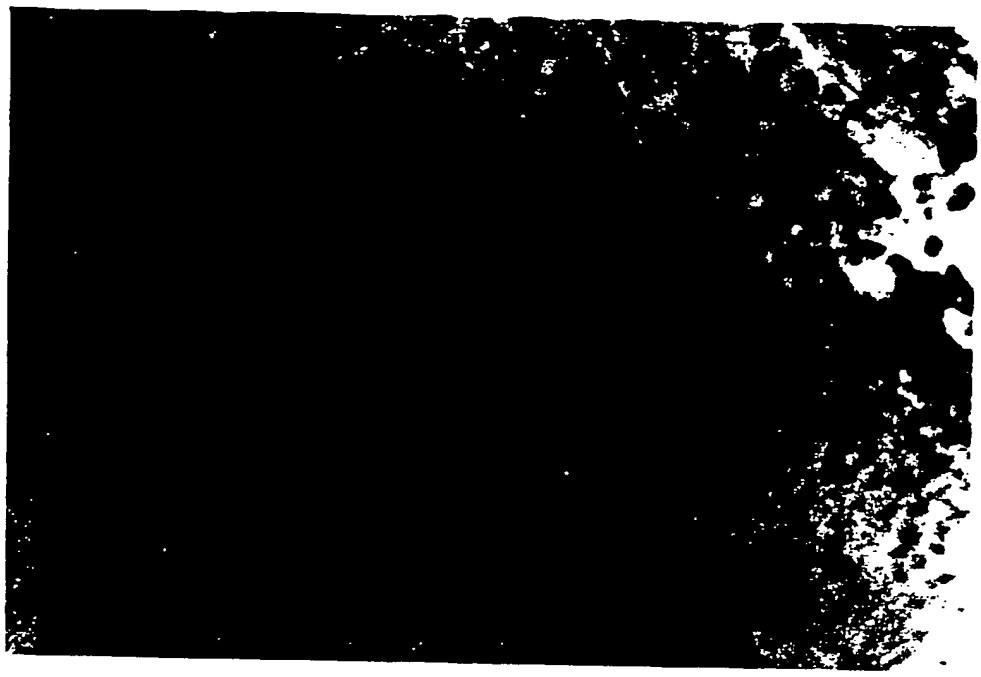


FIG.19a

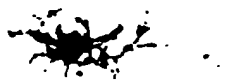
FIG.19b

FIG.19c

FIG.19d

a

FIG.20a



b

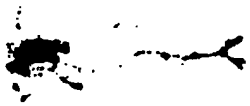


FIG. 20b

c

FIG.20c

FIG. 21a

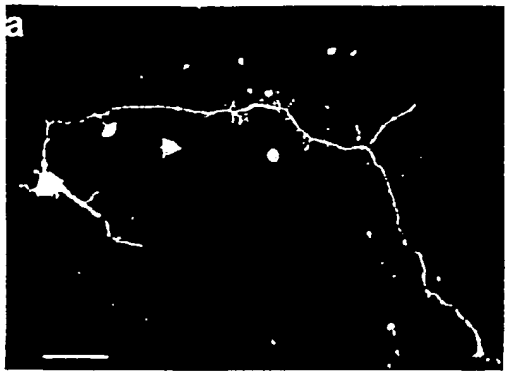
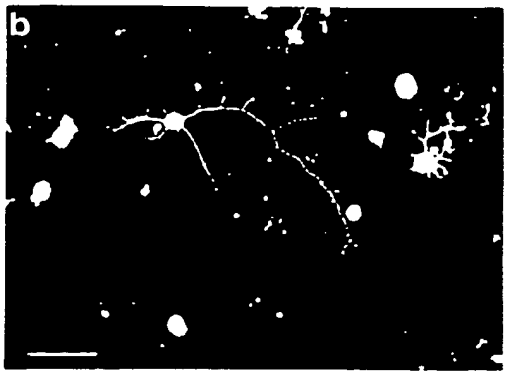


FIG. 21b



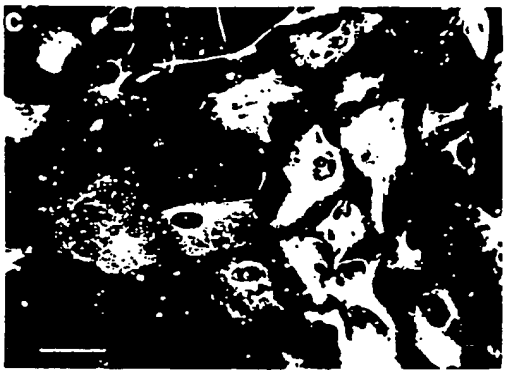


FIG. 21c

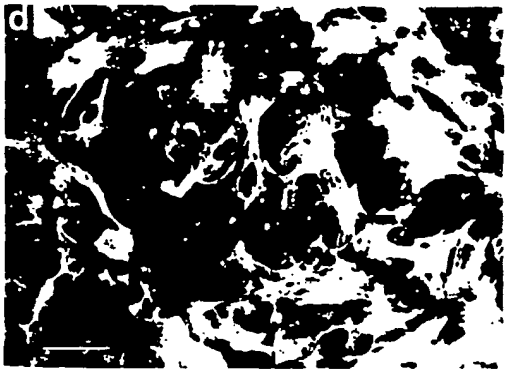


FIG. 21d

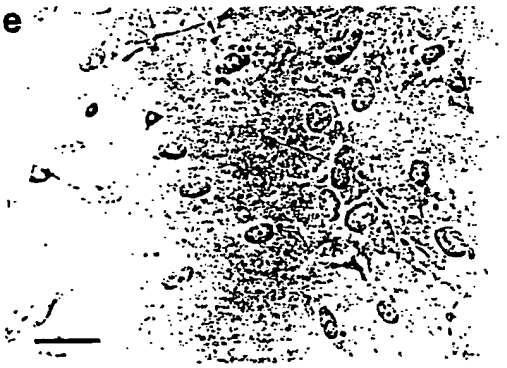


FIG. 21e

FIG. 21f

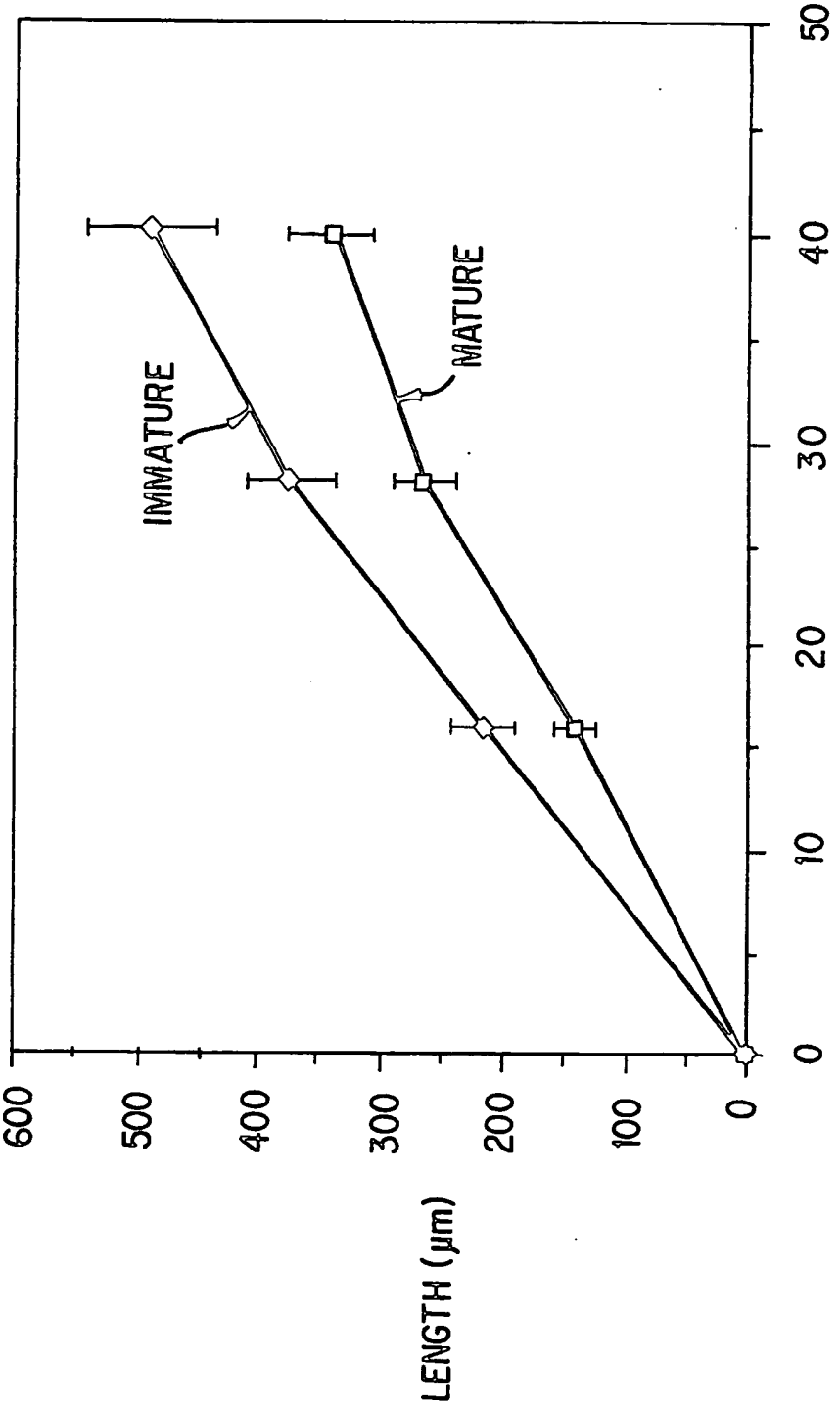

IMMATURE
MATURE
LENGTH (μm)
TIME (hr)
0
100
200
300
400
500
600
0
10
20
30
40
50

FIG. 22

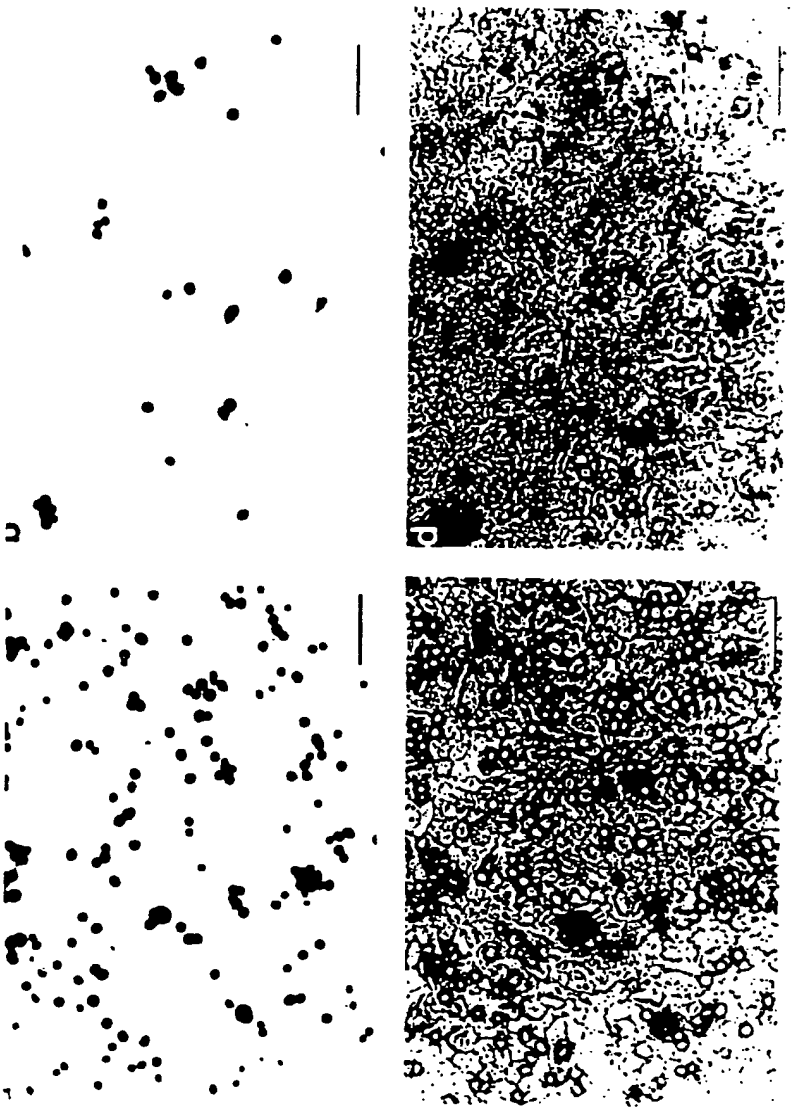


FIG. 23b

FIG. 23d

FIG. 23a

FIG. 23c

FIG. 24a

FIG. 24b

FIG. 24c

FIG. 24d

FIG. 25b

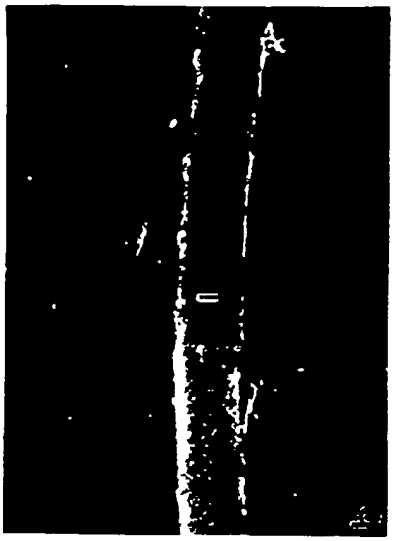


FIG. 25d

FIG. 25a

FIG. 25c

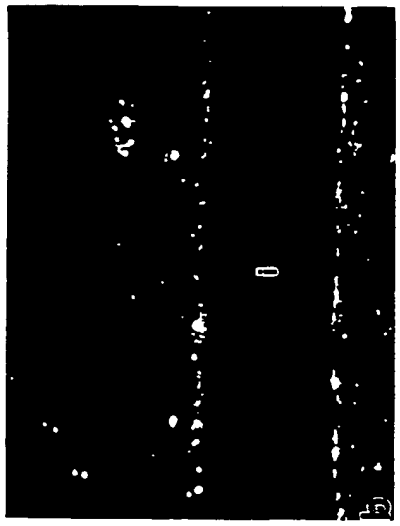


FIG. 26b

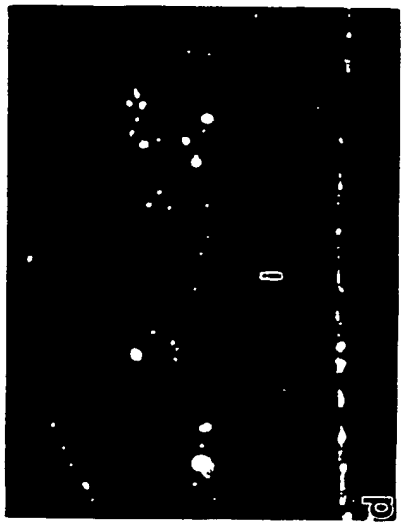


FIG. 26d

FIG. 26a

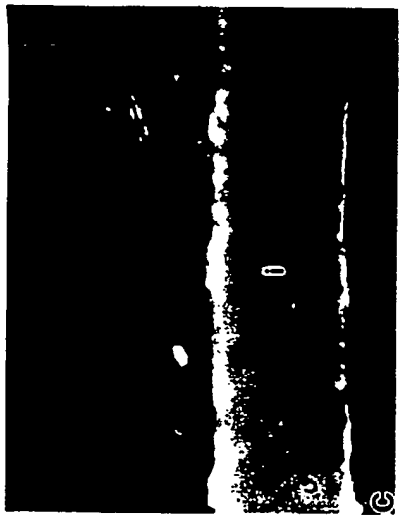


FIG. 26c

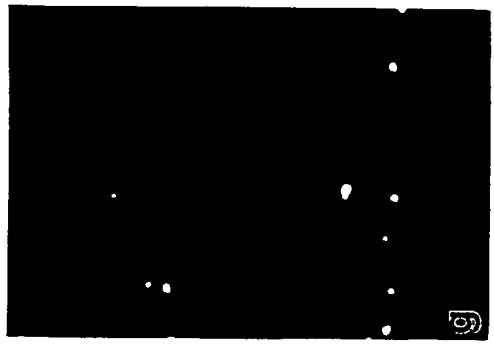


FIG. 26g

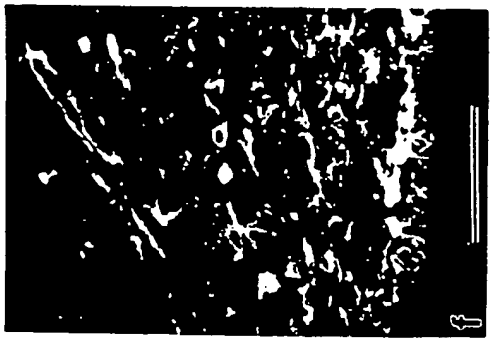


FIG. 26f

FIG. 26e

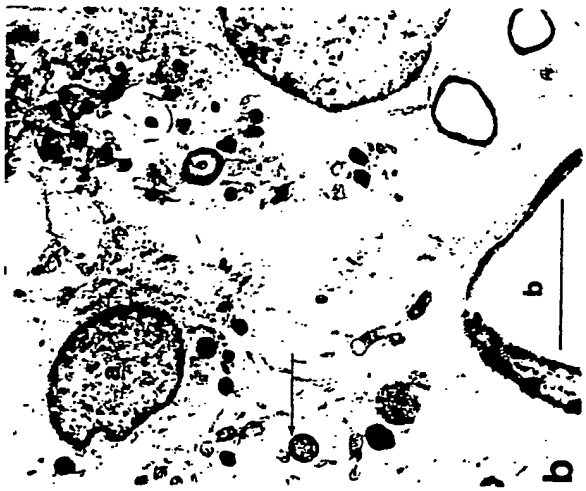


FIG. 27b

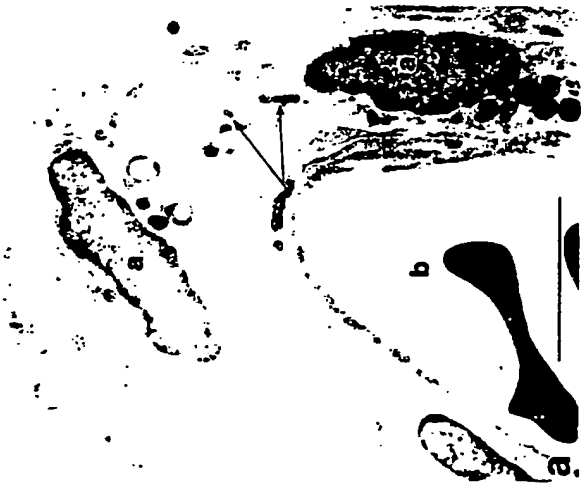


FIG. 27a

FIG. 27e

FIG. 27d

FIG. 27c

FIG. 28a

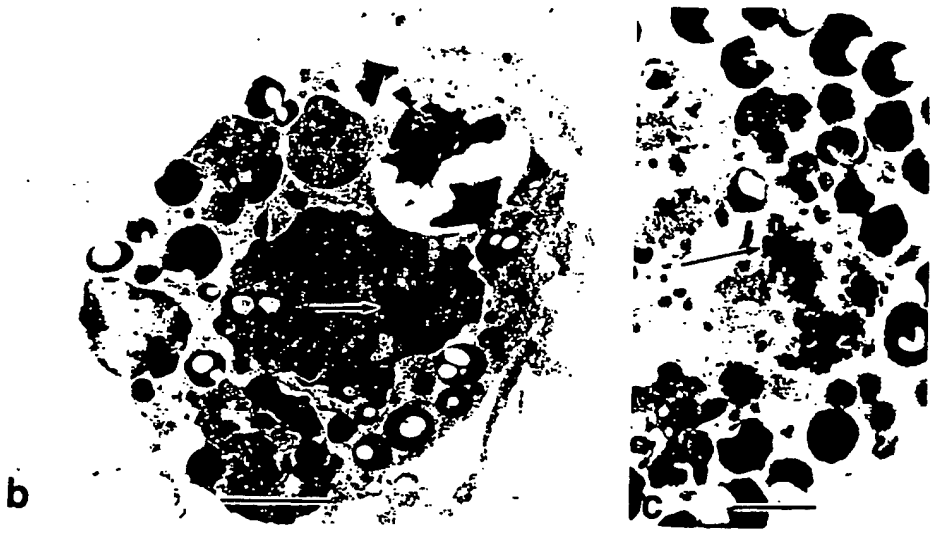


FIG. 28b FIG. 28c

FIG. 29a

FIG. 29b

FIG. 29c

FIG. 29d

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/06191

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 5/00; A61 K 37/00

U.S.: 424/570; 435/240.2, 240.26; 424/93, 425

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/93, 425, 570; 435/240.2, 240.26; 530/354; 514/801

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Biosis, Medline

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Clinical Research, Volume 36, No. 3, issued 1988, Silver, "Transplantation Strategies Using Embryonic Astoglial Cells to Promote CNS Axon Regeneration in Neonatal and Adult Mammals", pages 196-199, see the entire document.	29,30,33-36 1-16,23-28 31,32,37-40
X Y	Nature, Volume 325, issued 15 January 1987, Janzer et al, "Astrocytes induce blood-brain barrier properties in endothelial cells", pages 253-257, see the entire document.	29-31 17-25,33-34
Y	Biological Abstracts, Volume 79, No. 4 issued 1984, Doucette et al. "The glial cells in the nerve fiber layer of the rat olfactory bulb", see abstract no. 33497, Anat. Rec. 210(2), 385-392.	1,2,4-10, 12-16

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 January 1990	08 MAR 1991

International Searching Authority	Signature of Authorized Officer
ISA/US	J. M. Stone

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The Journal of Comparative Neurology, Volume 251, issued 1986, Smith et al, "Changing Role of Forebrain Astrocytes During Development, Regenerative Failure, and Induced Regeneration Upon Transplantation", pages 23-43, see the entire document.	1-16
Y	US, A, 4,707,448,(Major) 17 November 1987, see the entire document.	3,11,18,34